PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference 10430-WO	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/SE00/01449	International filing date *(day/month/year)* 06.07.2000	Priority date *(day/month/year)* 06.07.1999
International Patent Classification (IPC) or national classification and IPC7 C12Q 1/26, C12Q 1/28, G01N 33/12		
Applicant Forskarpatent i Syd AB et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of (6) 5 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
06.02.2001	24.10.2001
Name and mailing address of the IPEA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. 08-667 72 88	Authorized officer Hampus Rystedt/BS Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SE00/01449

I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed

☒ the description:
pages 1,4-15 , as originally filed
pages _____ , filed with the demand
pages 2,3 , filed with the letter of 01.08.2001

☒ the claims:
pages _____ , as originally filed
pages 16-18 , as amended (together with any statement) under article 19
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the drawings:
pages 1-3 , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☐ the sequence listing part of the description:
pages _____ , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages _____

☐ the claims, Nos. _____

☐ the drawings, sheet/fig _____

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2 (c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.*

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Box I.5

The amendment of a reference to Šebela et al. on page 3 is not allowed as this reference goes beyond the international application as originally filed. See Article 34 (b) PCT. This IPER has been established as if this amendment had not been made.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-12	YES
	Claims		NO
Inventive step (IS)	Claims	1-12	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-12	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

The following documents are considered relevant:

D1: IUBMB Enzyme nomenclature EC 1.4.3.6, URL http://www.chem.qmw.ac.uk/iubmb/enzyme/EC1/4/3/6.html, retrieved 2001-06-15
D2: Draisci, R. et al, Determination of biogenic amines with an electrochemical biosensor and its application to salted anchovies, Food chemistry, 1998, vol 62, pp 225-232
D3: Bouvrette, P. et al, Amperometric biosensor for diamine oxidase purified from porcine kidney, 1997, Enzyme and Microbial Technology, vol 20, pp 32-38
D4: Tombelli, S. and Mascini, M., Electrochemical biosensors for biogenic amines: a comparison of different approaches, 1998, Analytica Chimica Acta, vol 358, pp 277-284
D5: WO-A1-9323748
D6: US-A-5846702

The present application relate to a biosensor comprising an amine oxidase from grass pea.

The nomenclature of amine oxidases varies over publications. D1 shows that amine oxidase, monoamine oxidase and diamine oxidase is the same enzyme.

D2 discloses a mono-enzyme biosensor for determination of biogenic amines. The biosensor uses diamine oxidase from pea (*Cicer*). The enzymes are immobilised directly into membranes (polycarbonate or nylon) which are then attached to a platinum electrode

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Box V

D3 discloses a mono-enzyme biosensor for determination of biogenic amines. The biosensor uses diamine oxidase from pig. The diamine oxidase is immobilized into a Immunodyne membrane and attached to a glassy carbon electrode. Various electron mediators are also used.

D4 discloses both a mono-enzyme and a bi-enzyme biosensor for detection of biogenic amines. Amine oxidases from pig and pea (*Cicer arietinum)* and peroxidase from horseradish are used. The enzymes are either immobilized onto glass beads or into a cellulose acetate membrane (amine oxidase only).

D5 disclose the use of osmium-based redox polymers in enzyme electrodes. The electrodes may be of the mono-enzyme or bi-enzyme type and the enzymes may be immobilized either together in the redox polymer or in separate layers, see page 15 line 12 through page 17 line 13. D5 also states that graphite
D6 show different osmium based redox polymers for use in electrochemical biosensors. The redox polymer used in claim 5 of the present application is described in column 5 paragraph B and claim 20 of D6.

The claims amended under Article 19 PCT restrict the claimed scope to biosensors, and uses thereof, using amine oxidase from grass pea. This feature is novel compared to the prior art. As the applicant has shown that this enzyme gives the biosensor favourable characteristics compared to the biosensors of the prior art, claims 1-12 are considered inventive and also industrially applicable.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claim 1 characterizes a biosensor by the enzyme used. However, no reference is given to how this enzyme is obtained. The description thus do not comply with the requirement of Article 5 PCT that the description should described the invention in a manner sufficiently clear and complete for a person skilled in the art to carry out the invention.

CLAIMS

1. A biosensor for the detection and/or the determination of freshness biomarkers, such as biogenic amines, comprising an electrode and a mono-enzyme system, such as an amine oxidase, or a bi-enzyme system of an amine oxidase and a peroxidase.

2. The biosensor according to claim 1, **characterised in** that the amine oxidase is a copper containing amine oxidase.

3. The biosensor according to claim 1, **characterised in** that the bi-enzyme contains a copper containing amine oxidase coupled with a peroxidase a peroxidase, such as horseradish-, soybean-, tobacco-, sweet potato- or palmtree peroxidase.

4. The biosensor according to claim 3, **characterised in** that the peroxidase is horseradish peroxidase.

5. The biosensor according to any of claims 2 to 4, **characterised in** that the copper containing amine oxidase is derived from grass pea (AO, E.C. 1.4.3.6).

6. The biosensor according to any preceding of the claims, **characterised in** that the mono-enzyme- or the bi-enzyme- system is crosslinked into an osmium based redox polymer.

7. The biosensor according to claim 5, **characterised in** that the osmium based redox polymer includes poly(1-vinylimidazole) complexed with $[Os(4,4'\text{-dimetyl-bi-pyridin})_2 Cl]^{+/2+}$ and poly(etyleneglycol)diglycidyl-ether, as the crosslinking agent.

8. The biosensor according to any of the preceding claims, **characterised in** that the biosensor is of Type I, Type II or Type III type of biosensor; wherein

Type I: the mono-enzyme- or the bi-enzyme- system is added direct on to the electrode surface; or

Replaced by Article 34

Type II: the mono-enzyme- or the bi-enzyme- system is entrapped in the osmium based redox polymer added on the top of the electrode; or

Type III: the mono-enzyme- or the bi-enzyme- system and the osmium based redox polymer forms sequential coatings added on top of the electrode.

9. The biosensor according to claim 8, **characterised in** that the biosensor of Type III is one of Type III a, Type III b, Type III c or Type III d, wherein

Type III a: a second coating of the mono-enzyme is coating a dried layer of peroxidase and redox hydrogel; or

Type III b: a second coating of peroxidase and redox hydrogel is coating a dried layer of the mono-enzyme; or

Type III c: a second coating of the mono-enzyme entrapped in redox hydrogel is coating a dried layer of peroxidase; or

Type III d: a second coating of peroxidase is coating a dried layer of mono-enzyme entrapped in redox hydrogel.

10. The biosensor according to any preceding of the claims, **characterised in** that the electrode is of noble metals, such as gold, silver, platinum, palladium, or carbon/graphite-based material, such as graphite, carbon paste, vitrous carbon, carbon fibres, or conducting salts, or conducting polymers

11. The biosensor according to claim 10, **characterised in** that the electrode is made of graphite.

12. Use of the biosensor according to any of claims 1 to 11, as an analytical instrument or tool for the detection or determination of freshness biomarkers or of the content of

freshness biomarkers in food, such as meat from animals or fishes, or beverages.

13. Use of the biosensor according to any of claims 1 to 11, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in body fluids, such as blood, urine, saliva, sweat, in medical diagnoses or in the treatment of diseases.

14. Use of the biosensor according to any of claims 1 to 11, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in microdialysates or dialysates.

AMENDED CLAIMS

[received by the International Bureau on 7 March 2001 (07.03.01);
original claims 1-14 replaced by amended claims 1-12 (3 pages)]

1. A biosensor for the detection and/or the determination of freshness biomarkers, such as biogenic amines, comprising an electrode and a mono-enzyme system of an amine oxidase or a bi-enzyme system of an amine oxidase and a peroxidase, **characterised in** that the amine oxidase is a copper-containing amine oxidase derived from grass pea (AO, E.C. 1.4.3.6).

2. The biosensor according to claim 1, **characterised in** that the bi-enzyme system contains said copper-containing amine oxidase derived from grass pea coupled with horseradish, soy-bean, tobacco, sweet potato or palmtree peroxidase.

3. The biosensor according to claim 2, **characterised in** that the peroxidase is horseradish peroxidase.

4. The biosensor according to any of the preceding claims, **characterised in** that the mono-enzyme or the bi-enzyme system is crosslinked into an osmium based redox polymer.

5. The biosensor according to claim 4, **characterised in** that the osmium based redox polymer includes poly(1-vinyl-imidazole) complexed with $[Os(4,4'\text{-dimetyl-bi-pyridin})_2\ Cl]^{+/2+}$ and poly(etyleneglycol)diglycidyl-ether, as the crosslinking agent.

6. The biosensor according to any of the preceding claims, **characterised in** that the biosensor is of Type I, Type II or Type III type of biosensor; wherein

Type I: the mono-enzyme or the bi-enzyme system is added direct on to the electrode surface; or

Type II: the mono-enzyme or the bi-enzyme system is entrapped in the osmium based redox polymer added on the top of the electrode; or

Type III: the mono-enzyme or the bi-enzyme system and the osmium based redox polymer forms sequential coatings added on top of the electrode.

7. The biosensor according to claim 6, **characterised in** that the biosensor of Type III is one of Type III a, Type III b, Type III c or Type III d, wherein

Type III a: a second coating of the mono-enzyme is coating a dried layer of peroxidase and redox hydrogel; or

Type III b: a second coating of peroxidase and redox hydrogel is coating a dried layer of the mono-enzyme; or

Type III c: a second coating of the mono-enzyme entrapped in redox hydrogel is coating a dried layer of peroxidase; or

Type III d: a second coating of peroxidase is coating a dried layer of mono-enzyme entrapped in redox hydrogel.

8. The biosensor according to any of the preceding claims, **characterised in** that the electrode is of noble metals, such as gold, silver, platinum, palladium, or carbon/graphite-based material, such as graphite, carbon paste, vitrous carbon, carbon fibres, or conducting salts, or conducting polymers

9. The biosensor according to claim 8, **characterised in** that the electrode is made of graphite.

10. Use of the biosensor according to any of claims 1 to 9, as an analytical instrument or tool for the detection or determination of freshness biomarkers or of the content of freshness biomarkers in food, such as meat from animals or fishes, or beverages.

11. Use of the biosensor according to any of claims 1 to 9, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in body flu-

ids, such as blood, urine, saliva, sweat, in medical diagnoses or in the treatment of diseases.

12. Use of the biosensor according to any of claims 1 to 9, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in microdialysates or dialysates.

A preferred embodiment of the present invention is the biosensor comprising the mono-enzyme system comprising a copper containing amine oxidase (AO). The amine oxidase is preferably derived from grass pea (AO, E.C. 1.4.3.6).

Another preferred embodiment of the present invention is the biosensor comprising the bi-enzyme system comprising a copper containing amine oxidase (AO) coupled with a peroxidase (PO) such as horseradish (HRP), soybean, tobacco, sweet potato or palmtree peroxidase. The amine oxidase is preferably derived from grass pea (AO, E.C. 1.4.3.6).

Another preferred embodiment of the present invention the mono-enzyme- or the bi-enzyme- system is crosslinked into an osmium redox polymer. The osmium-based redox polymer is preferably (PVI_{13}-dmeOs) of poly- (1-vinyl-imidazole), complexed with $[Os-(4,4'\text{-dimetylbipyridine})_2\ Cl]^{+/2+}$, and a crosslinking agent such as poly-(ethyleneglycol)-diglycidyl-ether (PEGDGE).

Yet another embodiment of the present invention is the use of the biosensor as an analytical tool in the determination and/or detection of the freshness biomarkers in food.

Other uses and preferred embodiments of the present invention are defined in the use-claims and the subclaims.

DETAILED DESCRIPTION OF THE INVENTION

Amine oxidase represents a class of enzymes with a ubiquitous distribution in mammals, plants and micro-organisms. However, the structure, selectivity and biological functions are very different, depending on the isolation source. Grass-pea amine oxidase, fore instance, is a copper-containing amino oxidase, which besides the metal ions also contains an organic cofactor with a quinoide structure (topa-quinone) in its catalytic site.

In methods, where an amine oxidase is used, the enzyme is converting the amine to the corresponding aldehyde, with NH_3 and H_2O_2 release, according to reaction I

From US 5,565,329 is a method for determination of histamine concentration in a sample by determination of the decrease in dissolved oxygen (DO) known. The method involves adding a solution of an enzymatic reagent, which have a histamine oxidase activity, into an examination liquid containing the test sample and detect the sensor output signal. The analyser has a reaction cell provided with a DO electrode. The enzymatic reagent is a Cu-containing fungal amine oxidase. Which is extracted from a cellmass belonging to Aspergillus Niger cultured in a culture medium including amine as a nitrogen source. This approach is not very selective and sensitive.

Enzymatic determination of biogenic amines represents an alternative that can solve the above mentioned problems. However, most of the amino oxidase biosensors require a high operating potential (>500 mV vs. Ag/AgCl), which can lead to high background currents and low selectivity due to bias signals caused by electrochemically easily oxidisable interferences, which are always present in complex matrices, such as food or beverage.

SUMMERY OF THE INVENTION

As is clear from the description above a rapid, accurate, simple and handy analytical instrumental tool is needed for determination of food hygiene all along the food process line, starting from the source to the consumer.

With the present invention the above mentioned problems have been solved, the present invention offers a highly sensitive, selective rapid and very convenient determination and/or detection of the biomarkers in very small amounts.

Thus, the present invention relates to a biosensor for detection and/or determination of the content of freshness biomarkers in food or beverage. The biosensor comprises an electrode and a mono-enzyme system, e.g. amino oxidase, or a bi-enzyme system containing an amine oxidase coupled with a peroxidase.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 05 April 2001 (05.04.01)	
International application No. PCT/SE00/01449	**Applicant's or agent's file reference** 10430-WO
International filing date (day/month/year) 06 July 2000 (06.07.00)	**Priority date** (day/month/year) 06 July 1999 (06.07.99)
Applicant CSÖREGI, Elisabeth et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

06 February 2001 (06.02.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Charlotte ENGER
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

10430-WO

PCT REQUEST

Original (for **SUBMISSION**) - printed on 06.07.2000 01:42:10 PM

0 0-1	**For receiving Office use only** International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	

0-4 0-4-1	**Form - PCT/RO/101 PCT Request** Prepared using	PCT-EASY Version 2.90 (updated 10.05.2000)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	Swedish Patent Office (RO/SE)
0-7	**Applicant's or agent's file reference**	10430-WO
I	**Title of invention**	BIOSENSOR
II II-1 II-2 II-4 II-5 II-6 II-7 II-8 II-9	**Applicant** This person is: Applicant for Name Address: State of nationality State of residence Telephone No. Facsimile No.	applicant only all designated States except US FORSKARPATENT I SYD AB Ideon S-223 70 LUND Sweden SE +46 46 2863600 + 46 46 123472
III-1 III-1-1 III-1-2 III-1-4 III-1-5 III-1-6 III-1-7	**Applicant and/or inventor** This person is: Applicant for Name (LAST, First) Address: State of nationality State of residence	applicant and inventor US only CSÖREGI, Elisabeth Borgåsslingan 31 S-224 72 LUND Sweden SE

PCT REQUEST 10430-WO

Original (for **SUBMISSION**) - printed on 06.07.2000 01:42:10 PM

III-2	**Applicant and/or inventor**	
III-2-1	This person is:	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	NICULESCU, Mihaela
III-2-5	Address:	Systervägen 9 S-221 41 LUND Sweden
III-2-6	State of nationality	
III-2-7	State of residence	SE
III-3	**Applicant and/or inventor**	
III-3-1	This person is:	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	FRÉBORT, Ivo
III-3-5	Address:	Handkeho 5 779 00 OLOMOUC Czech Republic
III-3-6	State of nationality	
III-3-7	State of residence	CZ
IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	IVERSEN HASSELROT, Eva
IV-1-2	Address:	ALLIED ATTORNEYS CHEMICAL AB Box 24107 S-104 51 STOCKHOLM Sweden
IV-1-3	Telephone No.	+46 8 6627558
IV-1-4	Facsimile No.	+46 8 6607717
IV-1-5	e-mail	mail@alliedchem.se
IV-2	Additional agent(s)	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	FOGELBERG, Lennart; ONN, Staffan

PCT REQUEST

10430-WO

Original (for **SUBMISSION**) - printed on 06.07.2000 01:42:10 PM

V	**Designation of States**	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW MZ SD SL SZ TZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH&LI CN CR CU CZ DE DK DM DZ EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW
V-5	**Precautionary Designation Statement** In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.	
V-6	**Exclusion(s) from precautionary designations**	NONE
VI-1	**Priority claim of earlier national application**	
VI-1-1	Filing date	06 July 1999 (06.07.1999)
VI-1-2	Number	9902608-0
VI-1-3	Country	SE
VI-2	**Priority document request** The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1
VII-1	**International Searching Authority Chosen**	Swedish Patent Office (ISA/SE)

10430-WO

PCT REQUEST

Original (for **SUBMISSION**) - printed on 06.07.2000 01:42:10 PM

VIII	Check list	number of sheets	electronic file(s) attached
VIII-1	Request	4	-
VIII-2	Description	15	-
VIII-3	Claims	3	-
VIII-4	Abstract	1	10430-wo.txt
VIII-5	Drawings	2	-
VIII-7	TOTAL	25	
	Accompanying items	paper document(s) attached	electronic file(s) attached
VIII-8	Fee calculation sheet	✓	-
VIII-16	PCT-EASY diskette	-	diskette
VIII-18	**Figure of the drawings which should accompany the abstract**		
VIII-19	**Language of filing of the international application**	English	
IX-1	**Signature of applicant or agent**	Eva Iversen Hasselrot	
IX-1-1	Name (LAST, First)	IVERSEN HASSELROT, Eva	

FOR RECEIVING OFFICE USE ONLY

10-1	**Date of actual receipt of the purported international application**	
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/SE
10-6	**Transmittal of search copy delayed until search fee is paid**	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	**Date of receipt of the record copy by the International Bureau**	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01449

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/26, C12Q 1/28, G01N 33/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	M.NICULESCU ET AL: Redox Hydrogel-Based Amperometric Bienzyme Electrodes for Fish Freshness Monitoring; Anal. Chem., 72 (7), pages 1591 - 1597. Web Release Date: March 4, 2000.	1-14
	--	
P,X	M.NICULESCU ET AL: Amin Oxidase Based Amperometric Biosensors for Histamine Detection;Electroanalysis 2000, 12, No. 5, pages 369 -375	1,2,5-8, 10-14
P,Y		9
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 January 2001	26 -01- 2001
Name and mailing address of the ISA Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Hampus Rystedt/EÖ Telephone No. + 46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01449

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5565329 A (M. OHASHI ET AL), 15 October 1996 (15.10.96)	1,2,5,8, 12-14
Y		6-10
	--	
X	DIALOG(R)File 34: SciSearch (R); Accession No. 06515028. S.TOMBELLI ET AL: Electrochemical biosensors for biogenic amines: a comparison between different approaches; Analytica Chimica Acta, 1998, Vol.358, No.3 (Feb 10), pag. 277 - 284	1-5,8,10, 12-14
Y		6-10
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X	P.BOUVRETTE ET AL: Amperometric biosensor for diamine using diamine oxidase purified from porcine kidney; Enzyme and Microbial Technology, vol. 20, pag. 32 - 38	1,2,8,10-14
Y		6-10
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X	Sensors and Actuators B, Volume 32, 1996, G.C. Chemnitius et al, "Development of screen-printed enzyme electrodes for the estimation of fish quality" page 107 - page 113	1,2,5,8,10, 12-14
Y		6-10
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X	JOURNAL OF FOOD SCIENCE, Volume 61, No 5, 1996, KEITH B. MALE et al, "Amperometric Biosensor for Total Histamine, Putrescine and Cadaverine using Diamine Oxidase" page 1012 - page 1016	1,2,5,8,10, 12-14
Y		6-10
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01449

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Food Chemistry, Volume 62, No 2, 1998, R. Draisci et al, "Determination of biogenic amines with an electrochemical biosensor and its application to salted anchovies" page 225 - page 232	1,2,5,8,10, 12-14
Y	--	6-10
Y	WO 9323748 A1 (E. HELLER & COMPANY), 25 November 1993 (25.11.93), page 11, line 13 - page 12, line 17, page 9, lines 14-15, claims	6-10
	--	
Y	US 5846702 A (ZHI DAVID DENG ET AL), 8 December 1998 (08.12.98), see abstract, column 1, lines 14-29 and claims	6-9
	--	
Y	US 5378628 A (MICHAEL GRÄTZEL ET AL), 3 January 1995 (03.01.95), see abstract, table 1	6-9
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE00/01449

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE00/01449

According to Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. The present application relates to six such groups of inventions, namely:

1. An enzymatic biosensor for determining histamine concentrations using a mono-enzyme system and use thereof, according to claims 1, 2 and 5-14 (all partially).
2. An enzymatic biosensor for determining histamine concentrations using a bi-enzyme system and use thereof, according to claims 1-14 (all partially).
3. An enzymatic biosensor for determining histamine concentrations in which the enzymes are immobilised in an osmium based redox polymer and use thereof, according to claims 6-14 (all partially).
4. An enzymatic biosensor for determining histamine concentrations in which the enzymes are immobilised on the electrode surface and use thereof, according to claim 8-14 (all partially).
5. An enzymatic biosensor for determining histamine concentrations in which the enzymes and an osmium based redox polymer form sequential coatings on the top of the electrode and use thereof, according to claims 8-14 (all partially).
6. An enzymatic biosensor for determining histamine concentrations using an electrode made of noble metals, carbon or conducting salts or polymers and use thereof, according to claims 10-14 (all partially).

The technical feature common to all six inventions is a biosensor comprising an electrode and an enzyme system for detecting or determining biofreshness markers, e.g. histamine. This is well known in the prior art, through e.g. Male et al, Draisci et al, Chemnitius et al and Bouvrette et al cited in the preliminary search report. Consequently, this feature can not constitute the special technical feature required by Rule 13.2.

INTERNATIONAL SEARCH REPORT

Information on patent family members

27/12/00

International application No.
PCT/SE 00/01449

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		JP 5260993 A	12/10/93
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		DE 4392197 T	27/04/95
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US 5378628 A	03/01/95	AU 656360 B	02/02/95
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		WO 9214836 A	03/09/92

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WO 01/02827 A2

(54) Title: BIOSENSOR

(57) Abstract: The present invention relates to a biosensor for the detection and/or the determination of freshness biomarkers in food and beverage, comprising an electrode and a mono-enzyme system, such as an amine oxidase, or a bi-enzyme system of an amine oxidase and a peroxidase.

BIOSENSOR

The present invention relates to a biosensor, which includes an electrode and a mono-enzyme- or a bi-enzyme-system and uses of the biosensor.

BACKGROUND OF THE INVENTION

Rapid evaluation of food and beverage, such as fish, meat, quality is required in food industry. The biogenic amine content in food has been intensively studied because of their potential toxicity. Histamine is the most biologically active compound from this class, affecting the normal functions of the heart, smooth muscle, motor neurones, and gastric acid secretion. Other biogenic amines, such as putrescine and cadaverine, may amplify the effects caused by histamine intoxication, inhibiting the enzymes involved in histamine biodegradation: diamine oxidase and histamine-N-methyl transferase.

Numerous countries adopted maximum levels for histamine in food, especially in fish products. The Italian law has fixed a level of 100 mg/kg food, and similar limits have been adopted by EEC regulations.

Therefore, there is a need for developing of simple and inexpensive methods for determining of freshness biomarkers. Freshness biomarkers comprising inositol monophosphate, hypoxanthine and xanthine, these are intermediate degradation products of nucleic acids or biogenic amines, which are produced by microbial decarboxylation of the amino acids, histidine, ornithine, and lysine.

Classical methods for the determination of the content of biogenic amines are chromatographic techniques, such as gas chromatography, thin layer chromatography, reversed phase liquid chromatography, and liquid chromatography. However, these often require sample pre-treatment and relatively long analysing time, which leads to high costs and make these methods not suitable for routine use.

From US 5,565,329 is a method for determination of histamine concentration in a sample by determination of the decrease in dissolved oxygen (DO) known. The method involves adding a solution of an enzymatic reagent, which have a histamine oxidase activity, into an examination liquid containing the test sample and detect the sensor output signal. The analyser has a reaction cell provided with a DO electrode. The enzymatic reagent is a Cu-containing fungal amine oxidase. Which is extracted from a cellmass belonging to Aspergillus Niger cultured in a culture medium including amine as a nitrogen source. This approach is not very selective and sensitive.

Enzymatic determination of biogenic amines represents an alternative that can solve the above mentioned problems. However, most of the amino oxidase biosensors require a high operating potential (>500 mV vs. Ag/AgCl), which can lead to high background currents and low selectivity due to bias signals caused by electrochemically easily oxidisable interferences, which are always present in complex matrices, such as food or beverage.

SUMMERY OF THE INVENTION

As is clear from the description above a rapid, accurate, simple and handy analytical instrumental tool is needed for determination of food hygiene all along the food process line, starting from the source to the consumer.

With the present invention the above mentioned problems have been solved, the present invention offers a highly sensitive, selective rapid and very convenient determination and/or detection of the biomarkers in very small amounts.

Thus, the present invention relates to a biosensor for detection and/or determination of the content of freshness biomarkers in food or beverage. The biosensor comprises an electrode and a mono-enzyme system, e.g. amino oxidase, or a bi-enzyme system containing an amine oxidase coupled with a peroxidase.

A preferred embodiment of the present invention is the biosensor comprising the mono-enzyme system comprising a copper containing amine oxidase (AO). The amine oxidase is preferably derived from grass pea (AO, E.C. 1.4.3.6).

Another preferred embodiment of the present invention is the biosensor comprising the bi-enzyme system comprising a copper containing amine oxidase (AO) coupled with a peroxidase (PO) such as horseradish (HRP), soybean, tobacco, sweet potato or palmtree peroxidase. The amine oxidase is preferably derived from grass pea (AO, E.C. 1.4.3.6).

Another preferred embodiment of the present invention the mono-enzyme- or the bi-enzyme- system is crosslinked into an osmium redox polymer. The osmium-based redox polymer is preferably (PVI_{13}-dmeOs) of poly- (1-vinyl-imidazole), complexed with [Os-(4,4'-dimetylbipyridine)$_2$ Cl]$^{+/2+}$, and a crosslinking agent such as poly-(ethyleneglycol)-diglycidyl-ether (PEGDGE).

Yet another embodiment of the present invention is the use of the biosensor as an analytical tool in the determination and/or detection of the freshness biomarkers in food.

Other uses and preferred embodiments of the present invention are defined in the use-claims and the subclaims.

DETAILED DESCRIPTION OF THE INVENTION

Amine oxidase represents a class of enzymes with a ubiquitous distribution in mammals, plants and micro-organisms. However, the structure, selectivity and biological functions are very different, depending on the isolation source. Grass-pea amine oxidase, fore instance, is a copper-containing amino oxidase, which besides the metal ions also contains an organic cofactor with a quinoide structure (topa-quinone) in its catalytic site.

In methods, where an amine oxidase is used, the enzyme is converting the amine to the corresponding aldehyde, with NH_3 and H_2O_2 release, according to reaction I

$$R\text{-}CH_2\text{-}NR_2 + H_2O_2 + O_2 \rightarrow R\text{-}CHO + H_2O_2 + NH_3 \qquad I$$

Both oxygen consumption and hydrogen peroxide formation have been used for monitoring of biogenic amines on the basis of the above mentioned reaction.

It has surprisingly been shown that the interaction between the material of the electrode and the enzyme(s) resulted in a very selective and sensitive biosensor. The electrode has to be of any electron conducting material, such as noble metals, carbon/graphite-based material, conducting salts, conducting polymers etc.

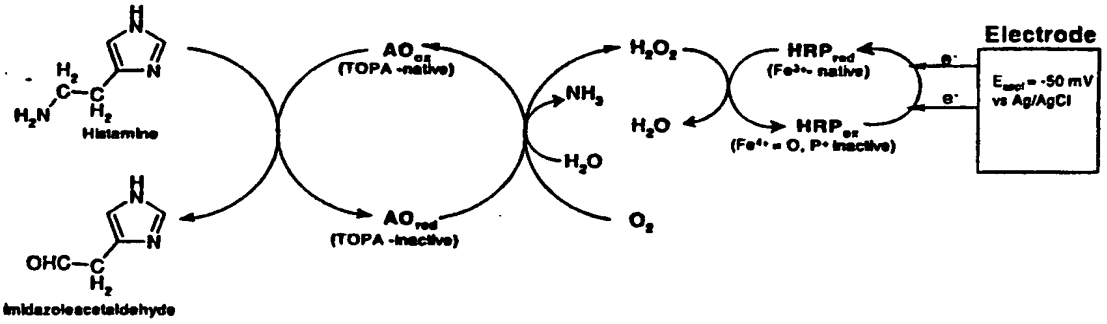
The mono-enzyme based biosensor according to the present invention is based either on the amine oxidase immobilised on top of an electrode (DET, direct electron transfer mechanism) or on amine oxidase crosslinked into a redox hydrogel forming a coating layer on top of an electrode (MET, mediated electron transfer mechanism).

According to the bi-enzymatic approach of the invention, the bi-enzyme electrode configuration is based on the enzyme amine oxidase (AO), from grass pea, and horseradish peroxidase (HRP) on a solid graphite electrode. The bi-enzymatic system is working at a potential where biases are minimal. The bi-enzyme electrodes were prepared either by simply adsorbing the two enzymes on the electrode surface (DET) or by crosslinking them into a redox polymer (MET). In the latter case the highly permeable and stable redox hydrogel is formed of a poly(1 - vinylimidazole) complexed with [Os(4,4'-dimetyl-bipyridine)$_2$Cl]$^{+/2+}$ (PV_{13}-dmeOs) and crosslinked to the enzymes by a crosslinking agent e.g. poly-(ethylene-glycol)-diglycidyl-ether (PEGDGE).

The optimal biosensor design was evaluated in terms of sensitivity, selectivity, life- and response-time, and it was used for the analysis of fish samples stored under different condi-

tions.

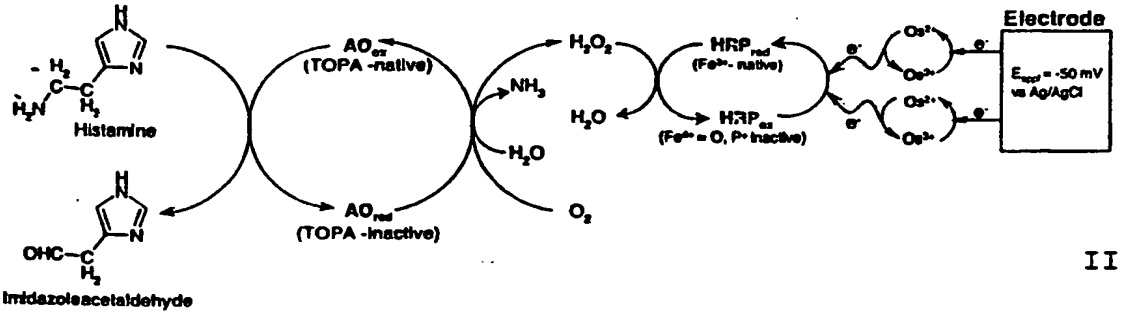
In the DET reaction mechanism, the biosensor of the present invention amine oxidase first converts the amine substrate (e.g. histamine) to an aldehyde product, the active form of the enzyme being recovered by oxidation of the organic cofactor in presence of molecular oxygen according to reaction mechanism II:



II

Then the active form of the enzyme being recovered by oxidation of the organic cofactor in presence of molecular oxygen, see mechanism II. The hydrogen peroxide formed during the first reaction is subsequently reduced to water by the action of peroxidase. The native form of the second enzyme is re-made either by direct reduction of its heme cofactor on the electrode surface or by receiving electrons from a mediator, maintained in it's reduced form by the potential applied on the graphite electrode (50 mV vs. Ag/AgCl).

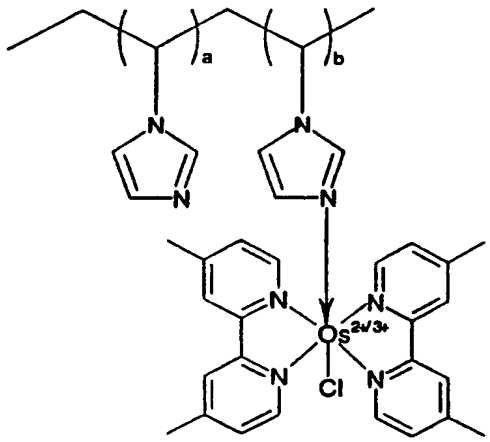
The peroxidase is either reduced by direct reduction of its heme cofactor (reaction mechanism II) or by receiving electrons from a mediator (MET), such as an osmium based redox polymer (see reaction mechanism III), maintained in its reduced form by the potential applied.



III

Redox hydro-gels are an effective matrix for enzyme immobilisation resulting in increased stability and the enhanced rates of the electron transfer. The rate of the electron transfer is highly influenced by the composition of the redox hydrogel, as well as by the kinetics of the used enzyme(s). Therefore various biosensor designs were considered in order to find the optimal electrode structure displaying the most efficient rate of electron transfer.

The structure of the redox polymer [Os(4,4'-dimetyl-bipyridine)$_2$Cl complexed to poly(1-vinyl-imidazole)] is shown in following formula:



The mono-enzyme- or the bi-enzyme- system is applied on to the electrode in three different ways (type I, II and III). In the following, enzyme means mono-enzyme- or bi-enzyme- system if not otherwise is stated.

Biosensor Type I: the enzyme is applied direct on to the electrode surface (DET). The reaction follows reaction mechanism II.

Biosensor Type II: the enzyme is entrapped in a redox hydrogel and applied on the top of the electrode (MET, one layer electrode). The reaction follows reaction mechanism III.

Biosensor Type III: represent a sequential coating procedure of enzyme and redox polymer (MET, bilayer electrode). The reaction

follows also reaction mechanism III.

In order to achieve an effective electron transfer all types of biosensors were optimised with regard to amount of immobilised enzyme and ratio of the used enzyme (Type I), composition of enzymes : redox polymer : crosslinking agent (Type II) and influence of electrode coating procedures (Type III).

PREPARATION OF BIOSENSORS

The biosensors were prepared by modifying graphite electrodes, which were prepared as follows:

i) Rods of spectroscopic graphite (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter) were cut, and polished on a wet fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI).

ii) The electrode surface was rinsed with water, dried at room temperature before coating with the enzymes. Three different electrode types were prepared:

Type I electrodes: were prepared by placing 6 µl of a premixed solution containing various amounts of AO (stock 20 mg/ml in phosphate buffer 0.1 M, pH 7.2 (PB)) and HRP (stock 10 mg/ml in PB) on the graphite electrode.

Type II electrodes: were prepared by cross-linking 6 µl of a mixture formed of AO (stock solution 20 mg/ml in PB), HRP (stock 10 mg/ml in PB) with an osmium redox hydrogel. The osmium redox hydrogel consisted of PV_{13}-dmeOS (stock 10 mg/ml in PB) and PEGDGE (5 mg/ml freshly prepared and used within 15 min). The bi-enzyme cross-linked into the redox hydrogel was placed on the top of the graphite electrode in different ratios in % by weight (w/w).

Type III electrodes: was prepared using a sequential coating procedure.

Type III a - first a premixed solution 6 µl of HRP_{13}-dmeOs, and PEGDGE was placed on the top of the electrode. Next, the electrodes were dried for 1 hour before coating with 6 µl of AO (see table III).

Type III b - first a solution of 6 µl of AO was placed on the top of the electrode. After drying for 1 hour, the electrodes were coated with 6 µl of a premixed solution of HRP, PV_{13}-dmeOs, and PEGDGE.

Type III c- in the first step, a drop of HRP solution (6 µl) was placed an the top of the electrode, and after its drying, a second layer containing 6 µl of a premixed solution of AO, PVI13-dmeOs, and PEGDGE was added.

Type III d - first a premixed solution of 6 µl of AO, PV_{13}-dmeOs, and PEGDGE was placed on the top of the electrode. Next, electrodes were dried for 1 hour before coating 6 µl of HRP.

If not otherwise stated, all modified electrodes were stored at 4°C for 14 h in a glass beaker and were rinsed with PB before use.

The bi-enzyme graphite electrodes were inserted as the working electrode in three electrode cell of wall jet-type placed in a single channel flow-injection system containing a manual sample injection valve (Valco Instruments Co. Inc., Houston, TX, USA) and a 50 µl injection loop.

A peristaltic pump (Alitea AB, Stockholm, Sweden) was used to pump the carrier solution at desired flow rates through Teflon tubings (0.5 mm i.d.) to the flow cell. A potentionstat (Zäta-Elektronik, Höör, Sweden) maintained the constant potential between the working and the reference electrode Ag/AgCl (0.1 M KCl). A platinum wire was used as the counter electrode. The response current was monitored with a single channel recorder (Model BD 111, Kipp&Zonen, Delft, The Netherlands).

Operational stability experiments were made using an Automated Sample Injection Analyser (Ismatec, Glattgurg-Zürich, Switzerland) by injecting samples of 100 µM histamine and 50 µM putrescine respectively, with a sample through-put of 30 injections/h using PB as the carrier solution at a flow rate of 0.5ml/min.

The increasing tendency of the apparent Michaelis-Menten constant with the amount of immobilised horseradish peroxidase was attributed to an increase in the thickness of the total protein loading on the electrode surface. The reducing the analytes diffusion rate in the film is effected by the influence of the protein loading. The maximum current, as well as the biosensors sensitivity trend show that the best combination is the one containing 80% by weight amine oxidase and 20% by weight horseradish peroxidase, which has been considered for the further experiments. The dynamic range for all the studied biosensors of Type I was 1 - 100 µM for both histamine and putrescine.

Different characteristics of Type I biosensors were measured and calculated for different ratios of amine oxidase AO and horseradish peroxidase HRP. The values are introduced into table I. Where I_{max} and K_m^{app} values are estimated from Michaelis-Menten equation:

$$I = (I_{max} \times [A]) / (K_m^{app} + [A])$$

In table I: A is analyte, S is the sensitivity, calculated as I_{max}/K_m^{app}, C is the conversion, calculated as $S_{analyte}/S_{H2O2}$ and DL is the detection limit, calculated as 3 S/N (signal- to -noise ratio).

TABLE I

Type of electrode (w/w)	Analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/Mcm^2)	C (%)	DL (μM)
AO 87%	Histamine	279±16	1.03±0.02	50.57±0.82	19.0	0.16
HRP 13%	Putrescine	153±15	1.96±0.06	175.48±1.40	66.2	0.06
	H_2O_2	93±3	1.80±0.21	265.13±1.65	-	-
AO 80%	Histamine	332±17	134±0.03	55.28±0.76	16.6	0.20
HRP 20%	Putrescine	228±15	3.01±0.07	180.84±0.95	54.7	0.07
	H_2O_2	112±8	2.07±0.06	330.23±1.02	-	-
AO 67%	Histamine	370±22	1.30±0.03	48.13±0.14	14.7	0.25
HRP 33%	Putrescine	240±15	3.10±0.01	176.94±0.87	54.2	0.70
	H_2O_2	153±6	3.64±0.04	325.90±0.56	-	-
AO 50%	Histamine	437±43	1.22±0.04	38.24±1.42	12.7	0.33
HRP 50%	Putrescine	268±23	3.05±0.10	155.90±1.26	52.0	0.08
	H_2O_2	175±8	3.83±0.05	299.80±0.65	-	-
AO 40%	Histamine	441±23	1.16±0.02	36.03±0.75	10.9	0.34
HRP 60%	Putrescine	276±22	3.69±0.06	183.14±1.11	55.7	0.13
	H_2O_2	206±3	4.94±0.03	328.50±0.22	-	-
AO 33%	Histamine	479±41	1.37±0.10	39.18±1.54	12.2	0.41
HRP 67%	Putrescine	287±12	3.84±0.06	183.28±0.61	57.0	0.08
	H_2O_2	211±18	4.95±0.15	321.36±1.24	-	-

Redox hydrogel based biosensors were optimised in order to determine the influence of the redox polycation and the crosslinking agent. Table II shows the obtained results.

If the diffusion barrier increased with the number of added components on the electrode surface, a tendency reflected in the change of the apparent Michaelis-Menten constants. K_M^{app} constant was increased with about 171% for histamine and 125% for putrescine. The introduction of the electrochemical mediator caused a considerable improvement in the bioelectrocatalytic efficiency, as can be seen from increase in the I_{max} with 262%

for histamine and 141% for putrescine and the sensitivity values with 33% for histamine and 7% for putrescine.

The hydrogen peroxide sensitivity remains practically unchanged. The detection limit and also the dynamic range for the studied analytes have also been improved in the case of type II electrodes.

In the table DR is the dynamic range and all the other symbols are the same as in Table I.

TABLE II

Type of electrode	Analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/Mcm^2)	C %	DL (μM)	DR (μM)
Type I	Histamine	332±17	1.34±0.02	55.29±0.73	16.74	0.16	1-100
	Putrescine	227±16	3.01±0.07	181.64±1.01	55.01	0.06	1-100
	H_2O_2	112±8	2.70±0.06	330.14±1.02			1-100
Type II	Histamine	901±85	4.85±0.41	3.874±1.73	23.07	0.33	1-150
	Putrescine	512±40	7.26±0.53	194.11±1.37	60.73	0.17	1-400
	H_2O_2	977±92	22.8±1.68	319.59±1.63			1-250

The effect of the coating procedure for the type II and type III biosensors was also studied. Besides coating with a premixed solutions of all four components, different possibilities of sequential coatings of the electrode surface, were also studied, see Table III. Both HRP and AO can be electrically wired to the redox polymer, and thus cause a partial short-circuit, when all components are mixed together. This was confirmed for the main substrate, putrescine, for which an increase in sensitivity of about 30 % was observed for the two layer electrodes (type III), compared to the one-layer electrodes (type II).

No considerable change was observed for the other substrate histamine, the slight decrease in sensitivity being not representative considering, the differences of about 10-15 % in

electrode preparation. Clearly, the less sensitive electrode configuration is represented by type III d type electrodes, for which the bias currents due to the wiring of AO are the most explicit. Considering the simplicity of electrode preparation and the small differences in the electrode characteristics between type II and type III electrodes, type II was chosen as optimal electrode design.

TABLE III

Type of electrode	Analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/Mcm^2)
Type II	Histamine	901±85	4.85±0.41	67.65±1.73
	Putrescine	512±40	7.26±0.53	194.24±1.46
Type IIIa	Histamine	789±35	3.56±0.08	61.80±0.68
	Putrescine	449±34	7.72±0.69	235.53±1.60
Type IIIb	Histamine	687±47	2.66±0.24	53.03±1.55
	Putrescine	473±28	2.04±0.13	59.08±1.19
Type IIIc	Histamine	689±33	2.17±0.06	43.14±0.75
	Putrescine	422±35	7.83±0.82	254.17±1.82
Type IIId	Histamine	649±19	1.90±0.02	40.10±0.42
	Putrescine	425±24	2.14±0.20	68.97±1.49

The influence of various components of the redox hydrogel on the biosensor characteristics is shown in Table IV. The increasing K_m^{app} in the presence of both PVI_{13}-dmeOs and PEGDGE demonstrated that the diffusion of the substrate was limited. This was because of the barrier formed by the mediator and/or cross-linking agent (rigidity of the redox hydrogel) on the surface of the electrode, which also resulted in an increased linear dynamic range. On the other hand, in the presence of crosslinked redox polycationic mediator (PVI_{13}-dmeOs), the I_{max} value was 100% increased suggesting that the final reduction step of the topa cofactor on the electrode surface is the rate-limiting step in the absence of the metyldiator.

In Table IV the response characteristics of different AO biosensors. The AO, PVI_{13}-dmeOs and PEGDGE concentrations were 5 mg/ml, 2 mg/ml and 0,5 mg/ml, respectively.

TABLE IV

Type of electrode	K_m^{app} (μM)	I_{max} (nA)	S (mA/Mcm^2)	DL (μM)	DR (μM)
AO	375±34	164±6.5	5.99±0.09	2.7	10-100
AO+PEGDGE	755±38	185±5.0	3.35±0.05	4.5	10-150
AO+OVI_{13}-dmeOs	770±14	235±2.4	4.18±0.02	3.7	10-150
AO+PVI_{13}-dmeOS+ PEGDGE	730±33	360±8.0	6.76±0.05	2.2	10-200

DESCRIPTION OF THE FIGURES

Figure 1: Shows a voltammogram for 100 μM histamine using an AO - HRP modified graphite electrode (I).

Figure 2: Shows the effect of the flow rate on the response current and sample throughput of Type I biosensors.

Figure 3: Shows the relative selectivity for different amine oxidase substrates, using histamine as reference compound, recorded for Type I (white) and Type II (black) electrodes.

Figure 4: Shows the monitoring of freshness in fish samples using Type II electrodes. The total amine concentration is expressed in histamine equivalent units.

The bi-enzyme electrodes were optimised with regard to several parameters, e.g. working potential, flow rate, influence of various enzyme ratios and electrode coating procedure.

Hydrodynamic voltammograms were recorded using 100 µM histamine as substrate and using an AO-HRP-modified type I electrodes in order to establish the optimal working potential. The voltammogram, together with the ratio between the response and the background current obtained in the same conditions, respectively, are shown in Figure 1. Although the response of the biosensor drastically increased when the applied potential was below -100mV so did the background current, which demonstrates a possible oxygen reduction interference with the biosensing process. A potential of -50 mV vs. Ag/AgCl was chosen as a compromise between the response and the background current. The background current obtained in the same condition (I_0), and the ratio between them (I/I_0). Conditions: electrode Type I, AO : HRP 1 : 1 (w/w), flow rate 0,5 ml/min.

The influence of carrier flow rate on the biosensor response for histamine was also considered for type I electrodes, the results are presented in Figure 2. The decrease in peak height with the increase in flow rate demonstrates a limitation due either to the bioconversion of the amine substrate by AO or to the reduction of H_2O_2 by the direct electron transfer between HRP and the graphite electrode. According to the obtained results an optimal working flow rate was chosen to be 0.5 ml/min, as a compromise between the biosensor kinetics and its sample throughput. Conditions: injections of 100 µM histamine, AO : HRP 1 : 1 (w/w), applied potential -50 mV vs. Ag/AgCl.

Type II biosensors were further characterised with regard to selectivity, response time, operational and storage stability. Figure 3 shows the relative selectivity for different AO substrates, using histamine as a reference compound, since it is a biomarker of major interest. As seen, the response for aliphatic amines is generally higher than those observed for the aromatic ones. Also, type II biosensors yielded higher sensitivities than type I ones, probably caused by better electron-transfer kinetics.

The response time of the sensor, calculated as the time elapsed between 5% and 95% of response height, was fast (less than 1 min).

The operational stability of the biosensor was studied both for histamine and putrescine as substrate. The response current of the bi-enzymatic enzyme electrode decreased with about 30% and 50% for histamine and putrescine, respectively. This after 10 h of continuos operation with a sample throughput of 30 injections/h. The storage stability of the electrodes was good, a decrease of only about 10% and 15% being observed fore histamine and putrescine, respectively, after 10 days of storage.

The substrates are histamine His, cystamine Cys, tyramine Tyr, spermidine Spr, etylenediamine EDA, agmatine Agm, putrescine Put, cadaverine Cad, Z-Ab-Z-1,4-diamino-2-butene and E-Ab-E-1,4-diamino-2-butene.

The optimised biosensor was considered for monitoring biogenetic amines in real samples. The differentiation between the signals given by different amines is not possible, only the total amine content in a sample could be determined. Triplets of 1.0 g of fish samples were taken from fish-muscles from trobot - *Psetta maxima* - and were kept in different conditions. The samples were homogenised in 10 ml PB. The homogenates were centrifuged at 13000 g for 60 min at 4°C. The supernatants were separated and immediately analysed by direct injection into the flow system. The fish-muscle samples, which had been kept both at 4°C and 25°C for 10 days, were analysed after extraction in PB by direct injection in the flow system. The total amine content expressed in histamine equivalents is presented in Figure 4. The maximum accepted limit for total amine concentration in food products is 100 to 200 mg/kg samples, and a concentration of 1000 mg/kg is considered to be toxic. After 3 days of storage at room temperature, the fish-samples become improper to consume, while even after 10 days of storage at 4°C there are not any major changes in the total amine concentration.

CLAIMS

1. A biosensor for the detection and/or the determination of freshness biomarkers, such as biogenic amines, comprising an electrode and a mono-enzyme system, such as an amine oxidase, or a bi-enzyme system of an amine oxidase and a peroxidase.

2. The biosensor according to claim 1, **characterised in** that the amine oxidase is a copper containing amine oxidase.

3. The biosensor according to claim 1, **characterised in** that the bi-enzyme contains a copper containing amine oxidase coupled with a peroxidase a peroxidase, such as horseradish-, soybean-, tobacco-, sweet potato- or palmtree peroxidase.

4. The biosensor according to claim 3, **characterised in** that the peroxidase is horseradish peroxidase.

5. The biosensor according to any of claims 2 to 4, **characterised in** that the copper containing amine oxidase is derived from grass pea (AO, E.C. 1.4.3.6).

6. The biosensor according to any preceding of the claims, **characterised in** that the mono-enzyme- or the bi-enzyme- system is crosslinked into an osmium based redox polymer.

7. The biosensor according to claim 5, **characterised in** that the osmium based redox polymer includes poly(1-vinylimidazole) complexed with $[Os(4,4'\text{-dimetyl-bi-pyridin})_2 Cl]^{+/2+}$ and poly(etyleneglycol)diglycidyl-ether, as the crosslinking agent.

8. The biosensor according to any of the preceding claims, **characterised in** that the biosensor is of Type I, Type II or Type III type of biosensor; wherein

Type I: the mono-enzyme- or the bi-enzyme- system is added direct on to the electrode surface; or

Type II: the mono-enzyme- or the bi-enzyme- system is entrapped in the osmium based redox polymer added on the top of the electrode; or

Type III: the mono-enzyme- or the bi-enzyme- system and the osmium based redox polymer forms sequential coatings added on top of the electrode.

9. The biosensor according to claim 8, **characterised in** that the biosensor of Type III is one of Type III a, Type III b, Type III c or Type III d, wherein

Type III a: a second coating of the mono-enzyme is coating a dried layer of peroxidase and redox hydrogel; or

Type III b: a second coating of peroxidase and redox hydrogel is coating a dried layer of the mono-enzyme; or

Type III c: a second coating of the mono-enzyme entrapped in redox hydrogel is coating a dried layer of peroxidase; or

Type III d: a second coating of peroxidase is coating a dried layer of mono-enzyme entrapped in redox hydrogel.

10. The biosensor according to any preceding of the claims, **characterised in** that the electrode is of noble metals, such as gold, silver, platinum, palladium, or carbon/graphite-based material, such as graphite, carbon paste, vitrous carbon, carbon fibres, or conducting salts, or conducting polymers

11. The biosensor according to claim 10, **characterised in** that the electrode is made of graphite.

12. Use of the biosensor according to any of claims 1 to 11, as an analytical instrument or tool for the detection or determination of freshness biomarkers or of the content of

freshness biomarkers in food, such as meat from animals or fishes, or beverages.

13. Use of the biosensor according to any of claims 1 to 11, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in body fluids, such as blood, urine, saliva, sweat, in medical diagnoses or in the treatment of diseases.

14. Use of the biosensor according to any of claims 1 to 11, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in microdialysates or dialysates.

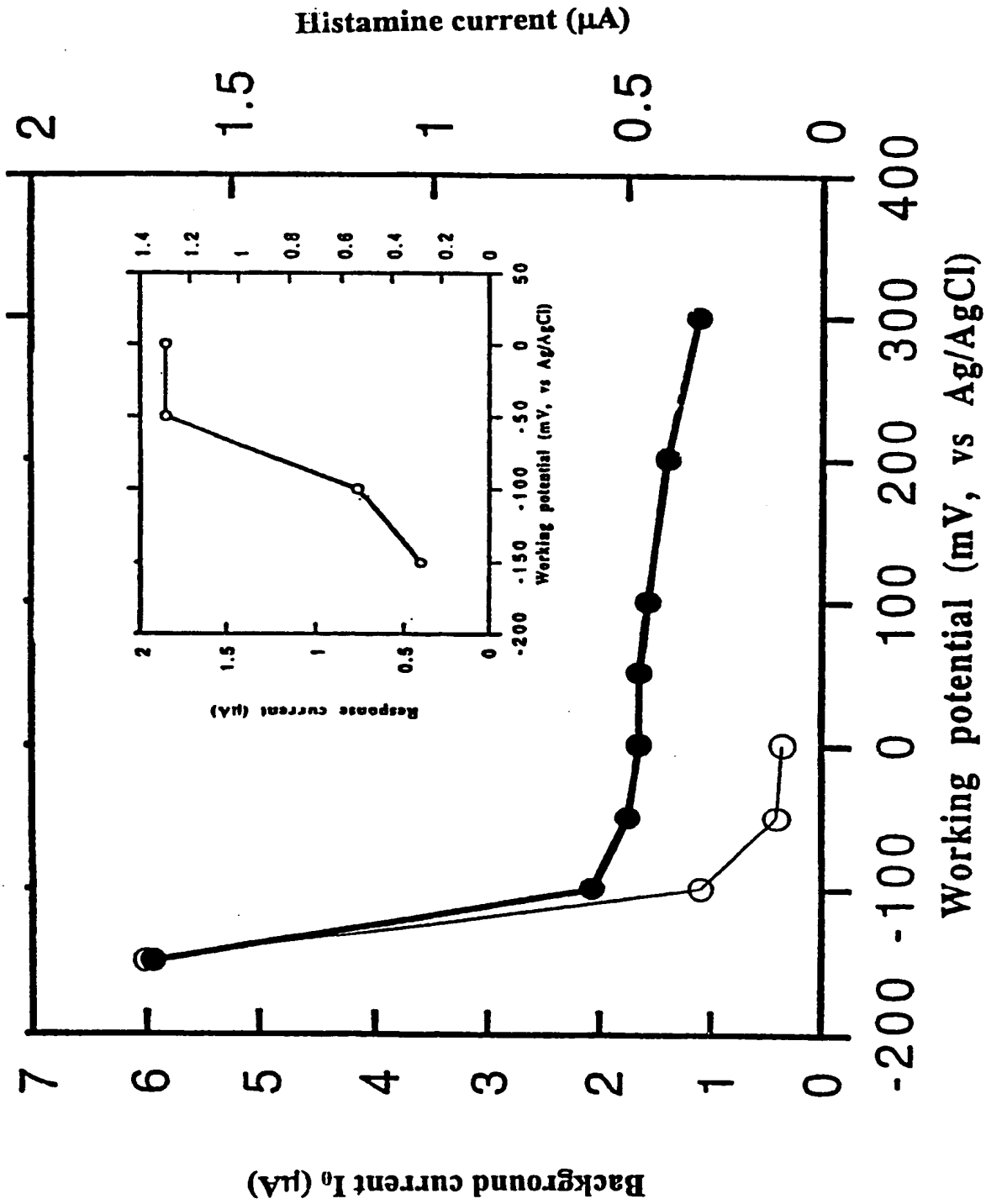

Histamine current (µA)
2
1.5
1
0.5
0
Working potential (mV, vs Ag/AgCl)
400
300
200
100
0
-100
-200
Background current I0 (µA)
7
6
5
4
3
2
1
0
Response current (µA)
Working potential (mV, vs Ag/AgCl)

Figure 1

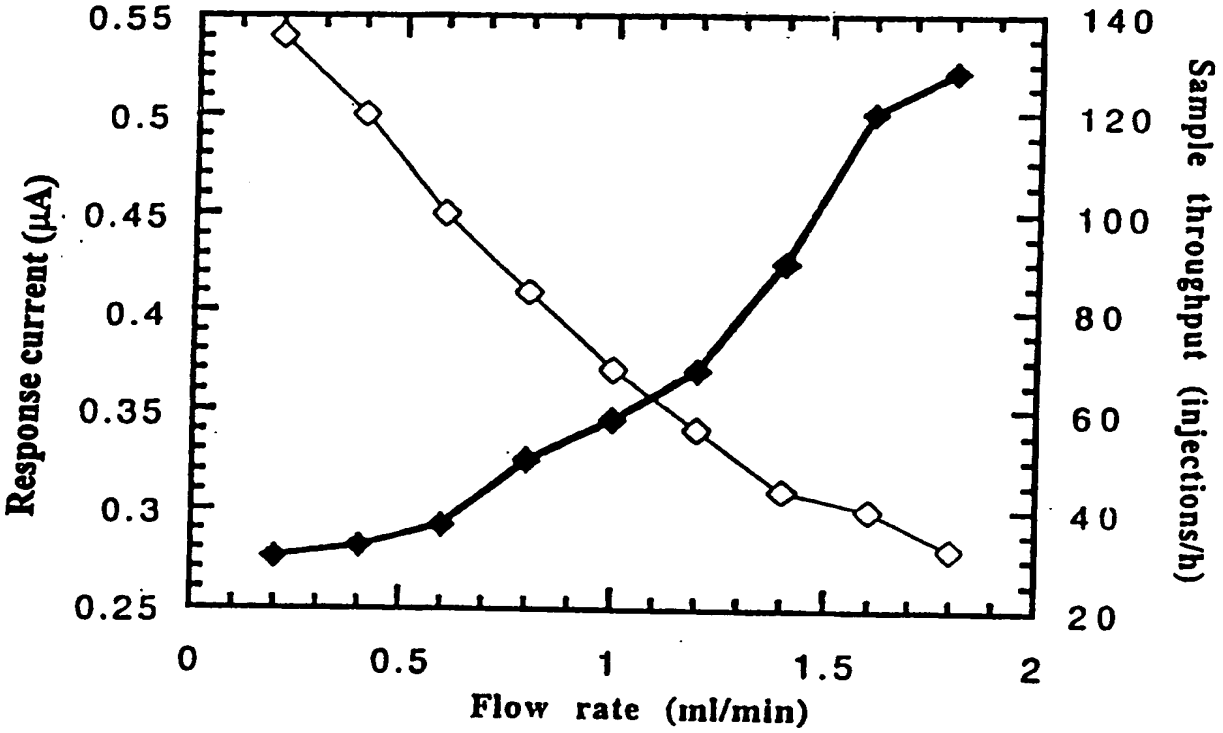


Figure 2

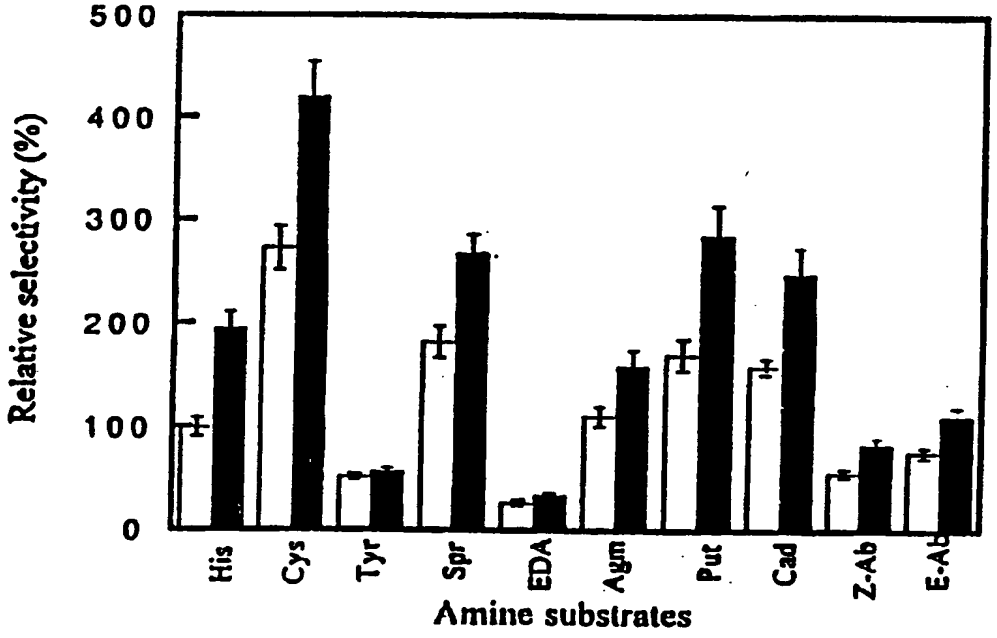


Figure 3

3/3

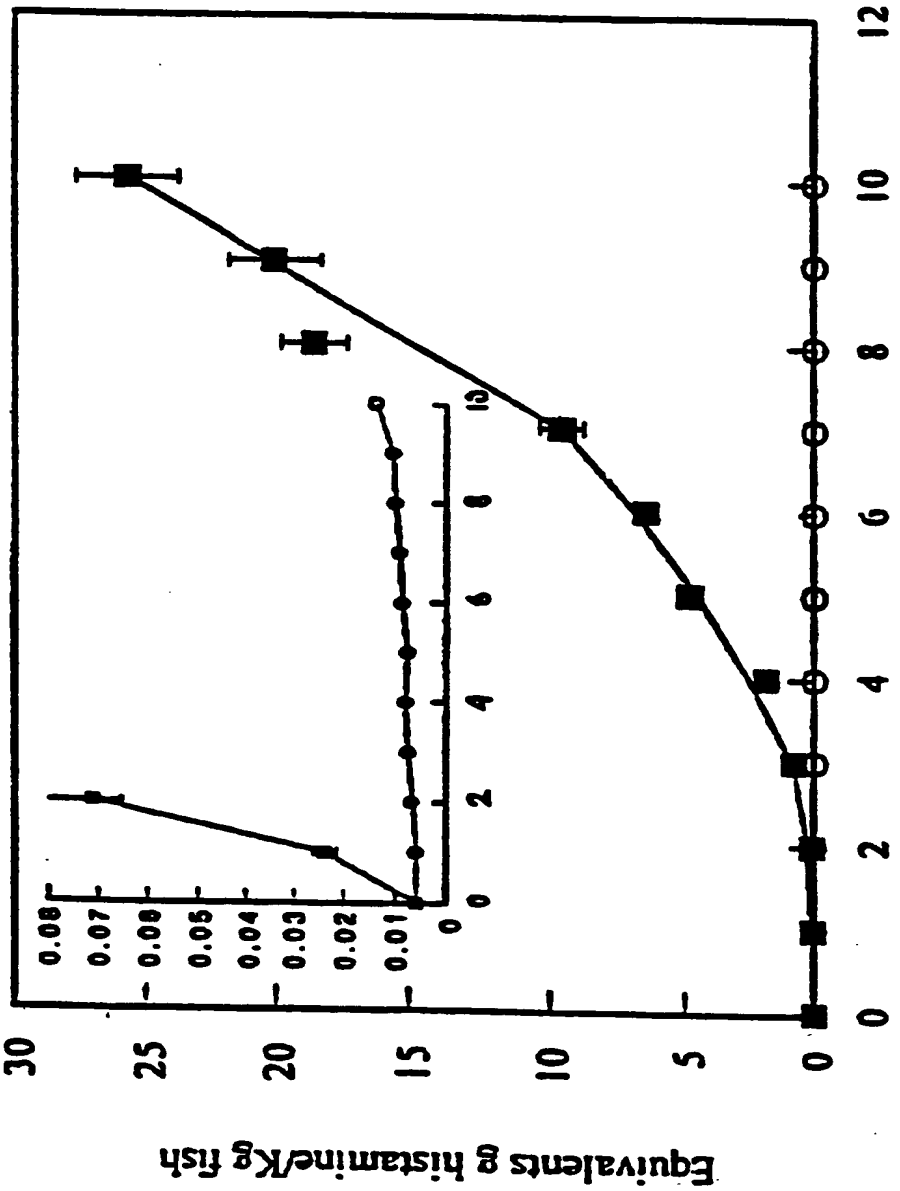

Equivalents g histamine/Kg fish
Time (days)
0
5
10
15
20
25
30
0
2
4
6
8
10
12
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08

Figure 4

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(75) Inventors/Applicants *(for US only)*: **CSÖREGI, Elisabeth** [SE/SE]; Borgåsslingan 31, S-224 72 Lund (SE). **NICULESCU, Mihaela** [SE/SE]; Systervägen 9, S-221 41 Lund (SE). **FRÉBORT, Ivo** [CZ/CZ]; Handkeho 5, 779 00 Olomouc (CZ).

(74) Agents: **IVERSEN HASSELROT, Eva** et al.; Allied Attorneys Chemical AB, Box 24107, S-104 51 Stockholm (SE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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— *With international search report.*
— *With amended claims.*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/02827 A3

(54) Title: BIOSENSOR

(57) Abstract: The present invention relates to a biosensor for the detection and/or the determination of freshness biomarkers, such as biogenic amines (preferably histamine) in food and beverage, comprising an electrode and a mono-enzyme system, such as an amine oxidase, or a bi-enzyme system of an amine oxidase and a peroxidase. The enzymes are optionally crosslinked into an osmium based redox polymer.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01449

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/26, C12Q 1/28, G01N 33/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	M.NICULESCU ET AL: Redox Hydrogel-Based Amperometric Bienzyme Electrodes for Fish Freshness Monitoring; Anal. Chem., 72 (7), pages 1591 - 1597. Web Release Date: March 4, 2000.	1-14
	--	
P,X	M.NICULESCU ET AL: Amin Oxidase Based Amperometric Biosensors for Histamine Detection;Electroanalysis 2000, 12, No. 5, pages 369 -375	1,2,5-8, 10-14
P,Y		9
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 January 2001	26 -01- 2001
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Hampus Rystedt/EÖ Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01449

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5565329 A (M. OHASHI ET AL), 15 October 1996 (15.10.96)	1,2,5,8, 12-14
Y		6-10
	--	
X	DIALOG(R)File 34: SciSearch (R); Accession No. 06515028. S.TOMBELLI ET AL: Electrochemical biosensors for biogenic amines: a comparison between different approaches; Analytica Chimica Acta, 1998, Vol.358, No.3 (Feb 10), pag. 277 - 284	1-5,8,10, 12-14
Y		6-10
	--	
X	P.BOUVRETTE ET AL: Amperometric biosensor for diamine using diamine oxidase purified from porcine kidney; Enzyme and Microbial Technology, vol. 20, pag. 32 - 38	1,2,8,10-14
Y		6-10
	--	
X	Sensors and Actuators B, Volume 32, 1996, G.C. Chemnitius et al, "Development of screen-printed enzyme electrodes for the estimation of fish quality" page 107 - page 113	1,2,5,8,10, 12-14
Y		6-10
	--	
X	JOURNAL OF FOOD SCIENCE, Volume 61, No 5, 1996, KEITH B. MALE et al, "Amperometric Biosensor for Total Histamine, Putrescine and Cadaverine using Diamine Oxidase" page 1012 - page 1016	1,2,5,8,10, 12-14
Y		6-10
	--	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01449

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Food Chemistry, Volume 62, No 2, 1998, R. Draisci et al, "Determination of biogenic amines with an electrochemical biosensor and its application to salted anchovies" page 225 - page 232	1,2,5,8,10, 12-14
Y	--	6-10
Y	WO 9323748 A1 (E. HELLER & COMPANY), 25 November 1993 (25.11.93), page 11, line 13 - page 12, line 17, page 9, lines 14-15, claims	6-10
	--	
Y	US 5846702 A (ZHI DAVID DENG ET AL), 8 December 1998 (08.12.98), see abstract, column 1, lines 14-29 and claims	6-9
	--	
Y	US 5378628 A (MICHAEL GRÄTZEL ET AL), 3 January 1995 (03.01.95), see abstract, table 1	6-9
	--	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE00/01449

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE00/01449

According to Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. The present application relates to six such groups of inventions, namely:

1. An enzymatic biosensor for determining histamine concentrations using a mono-enzyme system and use thereof, according to claims 1, 2 and 5-14 (all partially).
2. An enzymatic biosensor for determining histamine concentrations using a bi-enzyme system and use thereof, according to claims 1-14 (all partially).
3. An enzymatic biosensor for determining histamine concentrations in which the enzymes are immobilised in an osmium based redox polymer and use thereof, according to claims 6-14 (all partially).
4. An enzymatic biosensor for determining histamine concentrations in which the enzymes are immobilised on the electrode surface and use thereof, according to claim 8-14 (all partially).
5. An enzymatic biosensor for determining histamine concentrations in which the enzymes and an osmium based redox polymer form sequential coatings on the top of the electrode and use thereof, according to claims 8-14 (all partially).
6. An enzymatic biosensor for determining histamine concentrations using an electrode made of noble metals, carbon or conducting salts or polymers and use thereof, according to claims 10-14 (all partially).

The technical feature common to all six inventions is a biosensor comprising an electrode and an enzyme system for detecting or determining biofreshness markers, e.g. histamine. This is well known in the prior art, through e.g. Male et al, Draisci et al, Chemnitius et al and Bouvrette et al cited in the preliminary search report. Consequently, this feature can not constitute the special technical feature required by Rule 13.2.

INTERNATIONAL SEARCH REPORT

Information on patent family members

27/12/00

International application No.
PCT/SE 00/01449

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5565329 A	15/10/96	JP 2717745 B	25/02/98
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WO 9323748 A1	25/11/93	AU 3815593 A	13/12/93
		DE 4392197 T	27/04/95
		JP 7506675 T	20/07/95
		US 5320725 A	14/06/94
US 5846702 A	08/12/98	US 5589326 A	31/12/96
US 5378628 A	03/01/95	AU 656360 B	02/02/95
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		FR 2673289 A,B	28/08/92
		HU 66200 A	28/10/94
		HU 212451 B	28/06/96
		JP 2770250 B	25/06/98
		WO 9214836 A	03/09/92

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CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

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(51) International Patent Classification[7]: C12Q 1/26, 1/28, G01N 33/12

(21) International Application Number: PCT/SE00/01449

(22) International Filing Date: 6 July 2000 (06.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9902608-0 6 July 1999 (06.07.1999) SE

(71) Applicant *(for all designated States except US)*: **FORSKARPATENT I SYD AB** [SE/SE]; Ideon, S-223 70 Lund (SE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CSÖREGI, Elisabeth** [SE/SE]; Borgåsslingan 31, S-224 72 Lund (SE). **NICULESCU, Mihaela** [SE/SE]; Systervägen 9, S-221 41 Lund (SE). **FRÉBORT, Ivo** [CZ/CZ]; Handkeho 5, 779 00 Olomouc (CZ).

(74) Agents: **IVERSEN HASSELROT, Eva** et al.; Allied Attorneys Chemical AB, Box 24107, S-104 51 Stockholm (SE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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— *with amended claims*

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WO 01/02827 A3

(54) Title: BIOSENSOR

(57) Abstract: The present invention relates to a biosensor for the detection and/or the determination of freshness biomarkers, such as biogenic amines (preferably histamine) in food and beverage, comprising an electrode and a mono-enzyme system, such as an amine oxidase, or a bi-enzyme system of an amine oxidase and a peroxidase. The enzymes are optionally crosslinked into an osmium based redox polymer.

BIOSENSOR

The present invention relates to a biosensor, which includes an electrode and a mono-enzyme- or a bi-enzyme-system and uses of the biosensor.

BACKGROUND OF THE INVENTION

Rapid evaluation of food and beverage, such as fish, meat, quality is required in food industry. The biogenic amine content in food has been intensively studied because of their potential toxicity. Histamine is the most biologically active compound from this class, affecting the normal functions of the heart, smooth muscle, motor neurones, and gastric acid secretion. Other biogenic amines, such as putrescine and cadaverine, may amplify the effects caused by histamine intoxication, inhibiting the enzymes involved in histamine biodegradation: diamine oxidase and histamine-N-methyl transferase.

Numerous countries adopted maximum levels for histamine in food, especially in fish products. The Italian law has fixed a level of 100 mg/kg food, and similar limits have been adopted by EEC regulations.

Therefore, there is a need for developing of simple and inexpensive methods for determining of freshness biomarkers. Freshness biomarkers comprising inositol monophosphate, hypoxanthine and xanthine, these are intermediate degradation products of nucleic acids or biogenic amines, which are produced by microbial decarboxylation of the amino acids, histidine, ornithine, and lysine.

Classical methods for the determination of the content of biogenic amines are chromatographic techniques, such as gas chromatography, thin layer chromatography, reversed phase liquid chromatography, and liquid chromatography. However, these often require sample pre-treatment and relatively long analysing time, which leads to high costs and make these methods not suitable for routine use.

From US 5,565,329 is a method for determination of histamine concentration in a sample by determination of the decrease in dissolved oxygen (DO) known. The method involves adding a solution of an enzymatic reagent, which have a histamine oxidase activity, into an examination liquid containing the test sample and detect the sensor output signal. The analyser has a reaction cell provided with a DO electrode. The enzymatic reagent is a Cu-containing fungal amine oxidase. Which is extracted from a cellmass belonging to Aspergillus Niger cultured in a culture medium including amine as a nitrogen source. This approach is not very selective and sensitive.

Enzymatic determination of biogenic amines represents an alternative that can solve the above mentioned problems. However, most of the amino oxidase biosensors require a high operating potential (>500 mV vs. Ag/AgCl), which can lead to high background currents and low selectivity due to bias signals caused by electrochemically easily oxidisable interferences, which are always present in complex matrices, such as food or beverage.

SUMMERY OF THE INVENTION

As is clear from the description above a rapid, accurate, simple and handy analytical instrumental tool is needed for determination of food hygiene all along the food process line, starting from the source to the consumer.

With the present invention the above mentioned problems have been solved, the present invention offers a highly sensitive, selective rapid and very convenient determination and/or detection of the biomarkers in very small amounts.

Thus, the present invention relates to a biosensor for detection and/or determination of the content of freshness biomarkers in food or beverage. The biosensor comprises an electrode and a mono-enzyme system, e.g. amino oxidase, or a bi-enzyme system containing an amine oxidase coupled with a peroxidase.

A preferred embodiment of the present invention is the biosensor comprising the mono-enzyme system comprising a copper containing amine oxidase (AO). The amine oxidase is preferably derived from grass pea (AO, E.C. 1.4.3.6).

Another preferred embodiment of the present invention is the biosensor comprising the bi-enzyme system comprising a copper containing amine oxidase (AO) coupled with a peroxidase (PO) such as horseradish (HRP), soybean, tobacco, sweet potato or palmtree peroxidase. The amine oxidase is preferably derived from grass pea (AO, E.C. 1.4.3.6).

Another preferred embodiment of the present invention the mono-enzyme- or the bi-enzyme- system is crosslinked into an osmium redox polymer. The osmium-based redox polymer is preferably (PVI_{13}-dmeOs) of poly- (1-vinyl-imidazole), complexed with [Os-(4,4'-dimetylbipyridine)$_2$ Cl]$^{+/2+}$, and a crosslinking agent such as poly-(ethyleneglycol)-diglycidyl-ether (PEGDGE).

Yet another embodiment of the present invention is the use of the biosensor as an analytical tool in the determination and/or detection of the freshness biomarkers in food.

Other uses and preferred embodiments of the present invention are defined in the use-claims and the subclaims.

DETAILED DESCRIPTION OF THE INVENTION

Amine oxidase represents a class of enzymes with a ubiquitous distribution in mammals, plants and micro-organisms. However, the structure, selectivity and biological functions are very different, depending on the isolation source. Grass-pea amine oxidase, fore instance, is a copper-containing amino oxidase, which besides the metal ions also contains an organic cofactor with a quinoide structure (topa-quinone) in its catalytic site.

In methods, where an amine oxidase is used, the enzyme is converting the amine to the corresponding aldehyde, with NH_3 and H_2O_2 release, according to reaction I

$$R\text{-}CH_2\text{-}NR_2 + H_2O_2 + O_2 \rightarrow R\text{-}CHO + H_2O_2 + NH_3 \qquad I$$

Both oxygen consumption and hydrogen peroxide formation have been used for monitoring of biogenic amines on the basis of the above mentioned reaction.

It has surprisingly been shown that the interaction between the material of the electrode and the enzyme(s) resulted in a very selective and sensitive biosensor. The electrode has to be of any electron conducting material, such as noble metals, carbon/graphite-based material, conducting salts, conducting polymers etc.

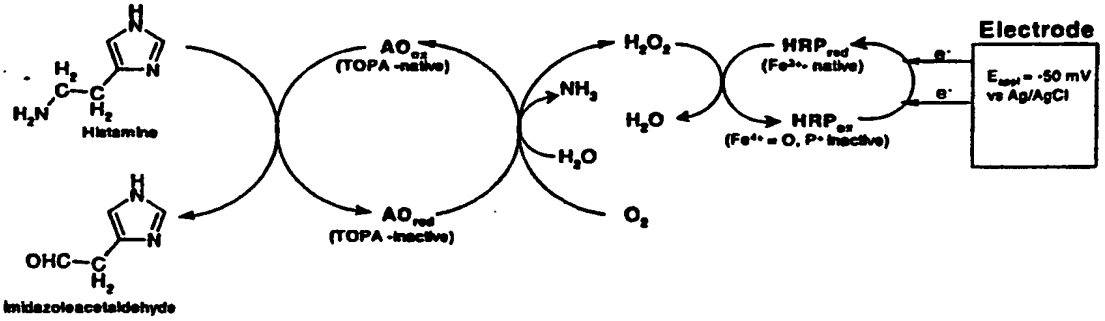
The mono-enzyme based biosensor according to the present invention is based either on the amine oxidase immobilised on top of an electrode (DET, direct electron transfer mechanism) or on amine oxidase crosslinked into a redox hydrogel forming a coating layer on top of an electrode (MET, mediated electron transfer mechanism).

According to the bi-enzymatic approach of the invention, the bi-enzyme electrode configuration is based on the enzyme amine oxidase (AO), from grass pea, and horseradish peroxidase (HRP) on a solid graphite electrode. The bi-enzymatic system is working at a potential where biases are minimal. The bi-enzyme electrodes were prepared either by simply adsorbing the two enzymes on the electrode surface (DET) or by crosslinking them into a redox polymer (MET). In the latter case the highly permeable and stable redox hydrogel is formed of a poly(1 - vinylimidazole) complexed with [Os(4,4'-dimetyl-bipyridine)$_2$Cl]$^{+/2+}$ (PV_{13}-dmeOs) and crosslinked to the enzymes by a crosslinking agent e.g. poly-(ethylene-glycol)-diglycidyl-ether (PEGDGE).

The optimal biosensor design was evaluated in terms of sensitivity, selectivity, life- and response-time, and it was used for the analysis of fish samples stored under different condi-

tions.

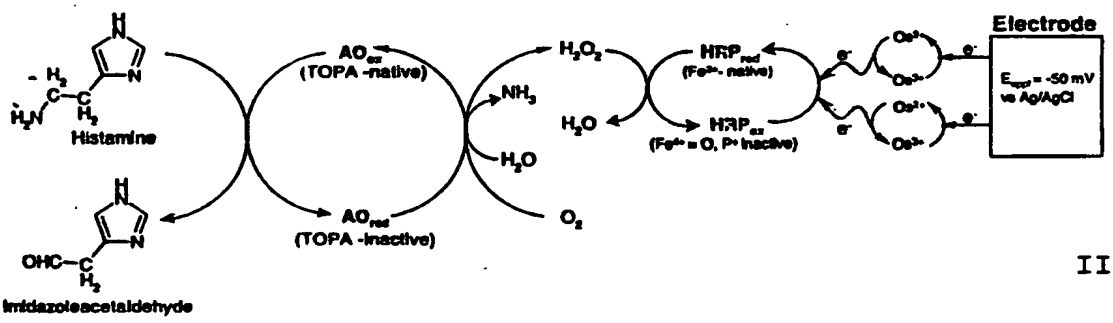
In the DET reaction mechanism, the biosensor of the present invention amine oxidase first converts the amine substrate (e.g. histamine) to an aldehyde product, the active form of the enzyme being recovered by oxidation of the organic cofactor in presence of molecular oxygen according to reaction mechanism II:



II

Then the active form of the enzyme being recovered by oxidation of the organic cofactor in presence of molecular oxygen, see mechanism II. The hydrogen peroxide formed during the first reaction is subsequently reduced to water by the action of peroxidase. The native form of the second enzyme is re-made either by direct reduction of its heme cofactor on the electrode surface or by receiving electrons from a mediator, maintained in it's reduced form by the potential applied on the graphite electrode (50 mV vs. Ag/AgCl).

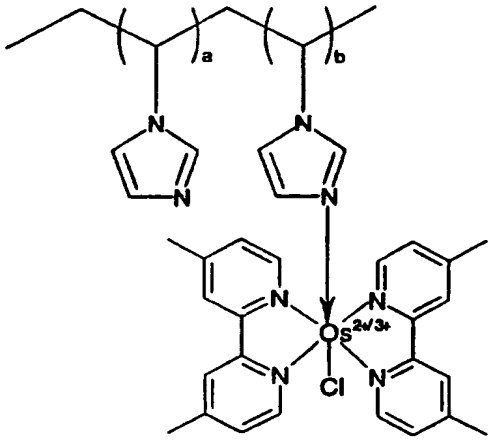
The peroxidase is either reduced by direct reduction of its heme cofactor (reaction mechanism II) or by receiving electrons from a mediator (MET), such as an osmium based redox polymer (see reaction mechanism III), maintained in its reduced form by the potential applied.



III

Redox hydro-gels are an effective matrix for enzyme immobilisation resulting in increased stability and the enhanced rates of the electron transfer. The rate of the electron transfer is highly influenced by the composition of the redox hydrogel, as well as by the kinetics of the used enzyme(s). Therefore various biosensor designs were considered in order to find the optimal electrode structure displaying the most efficient rate of electron transfer.

The structure of the redox polymer [Os(4,4'-dimetyl-bipyridine)$_2$Cl complexed to poly(1-vinyl-imidazole)] is shown in following formula:



The mono-enzyme- or the bi-enzyme- system is applied on to the electrode in three different ways (type I, II and III). In the following, enzyme means mono-enzyme- or bi-enzyme- system if not otherwise is stated.

Biosensor Type I: the enzyme is applied direct on to the electrode surface (DET). The reaction follows reaction mechanism II.

Biosensor Type II: the enzyme is entrapped in a redox hydrogel and applied on the top of the electrode (MET, one layer electrode). The reaction follows reaction mechanism III.

Biosensor Type III: represent a sequential coating procedure of enzyme and redox polymer (MET, bilayer electrode). The reaction

follows also reaction mechanism III.

In order to achieve an effective electron transfer all types of biosensors were optimised with regard to amount of immobilised enzyme and ratio of the used enzyme (Type I), composition of enzymes : redox polymer : crosslinking agent (Type II) and influence of electrode coating procedures (Type III).

PREPARATION OF BIOSENSORS

The biosensors were prepared by modifying graphite electrodes, which were prepared as follows:

i) Rods of spectroscopic graphite (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter) were cut, and polished on a wet fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI).

ii) The electrode surface was rinsed with water, dried at room temperature before coating with the enzymes. Three different electrode types were prepared:

Type I electrodes: were prepared by placing 6 µl of a premixed solution containing various amounts of AO (stock 20 mg/ml in phosphate buffer 0.1 M, pH 7.2 (PB)) and HRP (stock 10 mg/ml in PB) on the graphite electrode.

Type II electrodes: were prepared by cross-linking 6 µl of a mixture formed of AO (stock solution 20 mg/ml in PB), HRP (stock 10 mg/ml in PB) with an osmium redox hydrogel. The osmium redox hydrogel consisted of PV_{13}-dmeOS (stock 10 mg/ml in PB) and PEGDGE (5 mg/ml freshly prepared and used within 15 min). The bi-enzyme cross-linked into the redox hydrogel was placed on the top of the graphite electrode in different ratios in % by weight (w/w).

Type III electrodes: was prepared using a sequential coating procedure.

Type III a - first a premixed solution 6 µl of HRP_{13}-dmeOs, and PEGDGE was placed on the top of the electrode. Next, the electrodes were dried for 1 hour before coating with 6 µl of AO (see table III).

Type III b - first a solution of 6 µl of AO was placed on the top of the electrode. After drying for 1 hour, the electrodes were coated with 6 µl of a premixed solution of HRP, PV_{13}-dmeOs, and PEGDGE.

Type III c- in the first step, a drop of HRP solution (6 µl) was placed an the top of the electrode, and after its drying, a second layer containing 6 µl of a premixed solution of AO, PVI13-dmeOs, and PEGDGE was added.

Type III d - first a premixed solution of 6 µl of AO, PV_{13}-dmeOs, and PEGDGE was placed on the top of the electrode. Next, electrodes were dried for 1 hour before coating 6 µl of HRP.

If not otherwise stated, all modified electrodes were stored at 4°C for 14 h in a glass beaker and were rinsed with PB before use.

The bi-enzyme graphite electrodes were inserted as the working electrode in three electrode cell of wall jet-type placed in a single channel flow-injection system containing a manual sample injection valve (Valco Instruments Co. Inc., Houston, TX, USA) and a 50 µl injection loop.

A peristaltic pump (Alitea AB, Stockholm, Sweden) was used to pump the carrier solution at desired flow rates through Teflon tubings (0.5 mm i.d.) to the flow cell. A potentionstat (Zäta-Elektronik, Höör, Sweden) maintained the constant potential between the working and the reference electrode Ag/AgCl (0.1 M KCl). A platinum wire was used as the counter electrode. The response current was monitored with a single channel recorder (Model BD 111, Kipp&Zonen, Delft, The Netherlands).

Operational stability experiments were made using an Automated Sample Injection Analyser (Ismatec, Glattgurg-Zürich, Switzerland) by injecting samples of 100 µM histamine and 50 µM putrescine respectively, with a sample through-put of 30 injections/h using PB as the carrier solution at a flow rate of 0.5ml/min.

The increasing tendency of the apparent Michaelis-Menten constant with the amount of immobilised horseradish peroxidase was attributed to an increase in the thickness of the total protein loading on the electrode surface. The reducing the analytes diffusion rate in the film is effected by the influence of the protein loading. The maximum current, as well as the biosensors sensitivity trend show that the best combination is the one containing 80% by weight amine oxidase and 20% by weight horseradish peroxidase, which has been considered for the further experiments. The dynamic range for all the studied biosensors of Type I was 1 - 100 µM for both histamine and putrescine.

Different characteristics of Type I biosensors were measured and calculated for different ratios of amine oxidase AO and horseradish peroxidase HRP. The values are introduced into table I. Where I_{max} and K_m^{app} values are estimated from Michaelis-Menten equation:

$$I = (I_{max} \times [A]) / (K_m^{app} + [A])$$

In table I: A is analyte, S is the sensitivity, calculated as I_{max}/K_m^{app}, C is the conversion, calculated as $S_{analyte}/S_{H2O2}$ and DL is the detection limit, calculated as 3 S/N (signal- to -noise ratio).

TABLE I

Type of electrode (w/w)	Analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/Mcm^2)	C (%)	DL (μM)
AO 87%	Histamine	279±16	1.03±0.02	50.57±0.82	19.0	0.16
HRP 13%	Putrescine	153±15	1.96±0.06	175.48±1.40	66.2	0.06
	H_2O_2	93±3	1.80±0.21	265.13±1.65	-	-
AO 80%	Histamine	332±17	134±0.03	55.28±0.76	16.6	0.20
HRP 20%	Putrescine	228±15	3.01±0.07	180.84±0.95	54.7	0.07
	H_2O_2	112±8	2.07±0.06	330.23±1.02	-	-
AO 67%	Histamine	370±22	1.30±0.03	48.13±0.14	14.7	0.25
HRP 33%	Putrescine	240±15	3.10±0.01	176.94±0.87	54.2	0.70
	H_2O_2	153±6	3.64±0.04	325.90±0.56	-	-
AO 50%	Histamine	437±43	1.22±0.04	38.24±1.42	12.7	0.33
HRP 50%	Putrescine	268±23	3.05±0.10	155.90±1.26	52.0	0.08
	H_2O_2	175±8	3.83±0.05	299.80±0.65	-	-
AO 40%	Histamine	441±23	1.16±0.02	36.03±0.75	10.9	0.34
HRP 60%	Putrescine	276±22	3.69±0.06	183.14±1.11	55.7	0.13
	H_2O_2	206±3	4.94±0.03	328.50±0.22	-	-
AO 33%	Histamine	479±41	1.37±0.10	39.18±1.54	12.2	0.41
HRP 67%	Putrescine	287±12	3.84±0.06	183.28±0.61	57.0	0.08
	H_2O_2	211±18	4.95±0.15	321.36±1.24	-	-

Redox hydrogel based biosensors were optimised in order to determine the influence of the redox polycation and the crosslinking agent. Table II shows the obtained results.

If the diffusion barrier increased with the number of added components on the electrode surface, a tendency reflected in the change of the apparent Michaelis-Menten constants. K_M^{app} constant was increased with about 171% for histamine and 125% for putrescine. The introduction of the electrochemical mediator caused a considerable improvement in the bioelectrocatalytic efficiency, as can be seen from increase in the I_{max} with 262%

for histamine and 141% for putrescine and the sensitivity values with 33% for histamine and 7% for putrescine.

The hydrogen peroxide sensitivity remains practically unchanged. The detection limit and also the dynamic range for the studied analytes have also been improved in the case of type II electrodes.

In the table DR is the dynamic range and all the other symbols are the same as in Table I.

TABLE II

Type of electrode	Analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/Mcm^2)	C %	DL (μM)	DR (μM)
Type I	Histamine	332±17	1.34±0.02	55.29±0.73	16.74	0.16	1-100
	Putrescine	227±16	3.01±0.07	181.64±1.01	55.01	0.06	1-100
	H_2O_2	112±8	2.70±0.06	330.14±1.02			1-100
Type II	Histamine	901±85	4.85±0.41	3.874±1.73	23.07	0.33	1-150
	Putrescine	512±40	7.26±0.53	194.11±1.37	60.73	0.17	1-400
	H_2O_2	977±92	22.8±1.68	319.59±1.63			1-250

The effect of the coating procedure for the type II and type III biosensors was also studied. Besides coating with a premixed solutions of all four components, different possibilities of sequential coatings of the electrode surface, were also studied, see Table III. Both HRP and AO can be electrically wired to the redox polymer, and thus cause a partial short-circuit, when all components are mixed together. This was confirmed for the main substrate, putrescine, for which an increase in sensitivity of about 30 % was observed for the two layer electrodes (type III), compared to the one-layer electrodes (type II).

No considerable change was observed for the other substrate histamine, the slight decrease in sensitivity being not representative considering, the differences of about 10-15 % in

electrode preparation. Clearly, the less sensitive electrode configuration is represented by type III d type electrodes, for which the bias currents due to the wiring of AO are the most explicit. Considering the simplicity of electrode preparation and the small differences in the electrode characteristics between type II and type III electrodes, type II was chosen as optimal electrode design.

TABLE III

Type of electrode	Analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/Mcm^2)
Type II	Histamine	901±85	4.85±0.41	67.65±1.73
	Putrescine	512±40	7.26±0.53	194.24±1.46
Type IIIa	Histamine	789±35	3.56±0.08	61.80±0.68
	Putrescine	449±34	7.72±0.69	235.53±1.60
Type IIIb	Histamine	687±47	2.66±0.24	53.03±1.55
	Putrescine	473±28	2.04±0.13	59.08±1.19
Type IIIc	Histamine	689±33	2.17±0.06	43.14±0.75
	Putrescine	422±35	7.83±0.82	254.17±1.82
Type IIId	Histamine	649±19	1.90±0.02	40.10±0.42
	Putrescine	425±24	2.14±0.20	68.97±1.49

The influence of various components of the redox hydrogel on the biosensor characteristics is shown in Table IV. The increasing K_m^{app} in the presence of both PVI_{13}-dmeOs and PEGDGE demonstrated that the diffusion of the substrate was limited. This was because of the barrier formed by the mediator and/or cross-linking agent (rigidity of the redox hydrogel) on the surface of the electrode, which also resulted in an increased linear dynamic range. On the other hand, in the presence of crosslinked redox polycationic mediator (PVI_{13}-dmeOs), the I_{max} value was 100% increased suggesting that the final reduction step of the topa cofactor on the electrode surface is the rate-limiting step in the absence of the metyldiator.

In Table IV the response characteristics of different AO biosensors. The AO, PVI_{13}-dmeOs and PEGDGE concentrations were 5 mg/ml, 2 mg/ml and 0,5 mg/ml, respectively.

TABLE IV

Type of electrode	K_m^{app} (μM)	I_{max} (nA)	S (mA/Mcm^2)	DL (μM)	DR (μM)
AO	375±34	164±6.5	5.99±0.09	2.7	10-100
AO+PEGDGE	755±38	185±5.0	3.35±0.05	4.5	10-150
AO+OVI_{13}-dmeOs	770±14	235±2.4	4.18±0.02	3.7	10-150
AO+PVI_{13}-dmeOS+ PEGDGE	730±33	360±8.0	6.76±0.05	2.2	10-200

DESCRIPTION OF THE FIGURES

Figure 1: Shows a voltammogram for 100 μM histamine using an AO - HRP modified graphite electrode (I).

Figure 2: Shows the effect of the flow rate on the response current and sample throughput of Type I biosensors.

Figure 3: Shows the relative selectivity for different amine oxidase substrates, using histamine as reference compound, recorded for Type I (white) and Type II (black) electrodes.

Figure 4: Shows the monitoring of freshness in fish samples using Type II electrodes. The total amine concentration is expressed in histamine equivalent units.

The bi-enzyme electrodes were optimised with regard to several parameters, e.g. working potential, flow rate, influence of various enzyme ratios and electrode coating procedure.

Hydrodynamic voltammograms were recorded using 100 µM histamine as substrate and using an AO-HRP-modified type I electrodes in order to establish the optimal working potential. The voltammogram, together with the ratio between the response and the background current obtained in the same conditions, respectively, are shown in Figure 1. Although the response of the biosensor drastically increased when the applied potential was below -100mV so did the background current, which demonstrates a possible oxygen reduction interference with the biosensing process. A potential of -50 mV vs. Ag/AgCl was chosen as a compromise between the response and the background current. The background current obtained in the same condition (I_0), and the ratio between them (I/I_0). Conditions: electrode Type I, AO : HRP 1 : 1 (w/w), flow rate 0,5 ml/min.

The influence of carrier flow rate on the biosensor response for histamine was also considered for type I electrodes, the results are presented in Figure 2. The decrease in peak height with the increase in flow rate demonstrates a limitation due either to the bioconversion of the amine substrate by AO or to the reduction of H_2O_2 by the direct electron transfer between HRP and the graphite electrode. According to the obtained results an optimal working flow rate was chosen to be 0.5 ml/min, as a compromise between the biosensor kinetics and its sample throughput. Conditions: injections of 100 µM histamine, AO : HRP 1 : 1 (w/w), applied potential -50 mV vs. Ag/AgCl.

Type II biosensors were further characterised with regard to selectivity, response time, operational and storage stability. Figure 3 shows the relative selectivity for different AO substrates, using histamine as a reference compound, since it is a biomarker of major interest. As seen, the response for aliphatic amines is generally higher than those observed for the aromatic ones. Also, type II biosensors yielded higher sensitivities than type I ones, probably caused by better electron-transfer kinetics.

The response time of the sensor, calculated as the time elapsed between 5% and 95% of response height, was fast (less than 1 min).

The operational stability of the biosensor was studied both for histamine and putrescine as substrate. The response current of the bi-enzymatic enzyme electrode decreased with about 30% and 50% for histamine and putrescine, respectively. This after 10 h of continuos operation with a sample throughput of 30 injections/h. The storage stability of the electrodes was good, a decrease of only about 10% and 15% being observed fore histamine and putrescine, respectively, after 10 days of storage.

The substrates are histamine His, cystamine Cys, tyramine Tyr, spermidine Spr, etylenediamine EDA, agmatine Agm, putrescine Put, cadaverine Cad, Z-Ab-Z-1,4-diamino-2-butene and E-Ab-E-1,4-diamino-2-butene.

The optimised biosensor was considered for monitoring biogenetic amines in real samples. The differentiation between the signals given by different amines is not possible, only the total amine content in a sample could be determined. Triplets of 1.0 g of fish samples were taken from fish-muscles from trobot - *Psetta maxima* - and were kept in different conditions. The samples were homogenised in 10 ml PB. The homogenates were centrifuged at 13000 g for 60 min at 4°C. The supernatants were separated and immediately analysed by direct injection into the flow system. The fish-muscle samples, which had been kept both at 4°C and 25°C for 10 days, were analysed after extraction in PB by direct injection in the flow system. The total amine content expressed in histamine equivalents is presented in Figure 4. The maximum accepted limit for total amine concentration in food products is 100 to 200 mg/kg samples, and a concentration of 1000 mg/kg is considered to be toxic. After 3 days of storage at room temperature, the fish-samples become improper to consume, while even after 10 days of storage at 4°C there are not any major changes in the total amine concentration.

CLAIMS

1. A biosensor for the detection and/or the determination of freshness biomarkers, such as biogenic amines, comprising an electrode and a mono-enzyme system, such as an amine oxidase, or a bi-enzyme system of an amine oxidase and a peroxidase.

2. The biosensor according to claim 1, **characterised in** that the amine oxidase is a copper containing amine oxidase.

3. The biosensor according to claim 1, **characterised in** that the bi-enzyme contains a copper containing amine oxidase coupled with a peroxidase a peroxidase, such as horseradish-, soybean-, tobacco-, sweet potato- or palmtree peroxidase.

4. The biosensor according to claim 3, **characterised in** that the peroxidase is horseradish peroxidase.

5. The biosensor according to any of claims 2 to 4, **characterised in** that the copper containing amine oxidase is derived from grass pea (AO, E.C. 1.4.3.6).

6. The biosensor according to any preceding of the claims, **characterised in** that the mono-enzyme- or the bi-enzyme- system is crosslinked into an osmium based redox polymer.

7. The biosensor according to claim 5, **characterised in** that the osmium based redox polymer includes poly(1-vinylimidazole) complexed with $[Os(4,4'\text{-dimetyl-bi-pyridin})_2 Cl]^{+/2+}$ and poly(etyleneglycol)diglycidyl-ether, as the crosslinking agent.

8. The biosensor according to any of the preceding claims, **characterised in** that the biosensor is of Type I, Type II or Type III type of biosensor; wherein

Type I: the mono-enzyme- or the bi-enzyme- system is added direct on to the electrode surface; or

Type II: the mono-enzyme- or the bi-enzyme- system is entrapped in the osmium based redox polymer added on the top of the electrode; or

Type III: the mono-enzyme- or the bi-enzyme- system and the osmium based redox polymer forms sequential coatings added on top of the electrode.

9. The biosensor according to claim 8, **characterised in** that the biosensor of Type III is one of Type III a, Type III b, Type III c or Type III d, wherein

Type III a: a second coating of the mono-enzyme is coating a dried layer of peroxidase and redox hydrogel; or

Type III b: a second coating of peroxidase and redox hydrogel is coating a dried layer of the mono-enzyme; or

Type III c: a second coating of the mono-enzyme entrapped in redox hydrogel is coating a dried layer of peroxidase; or

Type III d: a second coating of peroxidase is coating a dried layer of mono-enzyme entrapped in redox hydrogel.

10. The biosensor according to any preceding of the claims, **characterised in** that the electrode is of noble metals, such as gold, silver, platinum, palladium, or carbon/graphite-based material, such as graphite, carbon paste, vitrous carbon, carbon fibres, or conducting salts, or conducting polymers

11. The biosensor according to claim 10, **characterised in** that the electrode is made of graphite.

12. Use of the biosensor according to any of claims 1 to 11, as an analytical instrument or tool for the detection or determination of freshness biomarkers or of the content of

freshness biomarkers in food, such as meat from animals or fishes, or beverages.

13. Use of the biosensor according to any of claims 1 to 11, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in body fluids, such as blood, urine, saliva, sweat, in medical diagnoses or in the treatment of diseases.

14. Use of the biosensor according to any of claims 1 to 11, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in microdialysates or dialysates.

AMENDED CLAIMS

[received by the International Bureau on 7 March 2001 (07.03.01); original claims 1-14 replaced by amended claims 1-12 (3 pages)]

1. A biosensor for the detection and/or the determination of freshness biomarkers, such as biogenic amines, comprising an electrode and a mono-enzyme system of an amine oxidase or a bi-enzyme system of an amine oxidase and a peroxidase, **characterised in** that the amine oxidase is a copper-containing amine oxidase derived from grass pea (AO, E.C. 1.4.3.6).

2. The biosensor according to claim 1, **characterised in** that the bi-enzyme system contains said copper-containing amine oxidase derived from grass pea coupled with horseradish, soybean, tobacco, sweet potato or palmtree peroxidase.

3. The biosensor according to claim 2, **characterised in** that the peroxidase is horseradish peroxidase.

4. The biosensor according to any of the preceding claims, **characterised in** that the mono-enzyme or the bi-enzyme system is crosslinked into an osmium based redox polymer.

5. The biosensor according to claim 4, **characterised in** that the osmium based redox polymer includes poly(1-vinyl-imidazole) complexed with $[Os(4,4'\text{-dimetyl-bi-pyridin})_2\ Cl]^{+/2+}$ and poly(etyleneglycol)diglycidyl-ether, as the crosslinking agent.

6. The biosensor according to any of the preceding claims, **characterised in** that the biosensor is of Type I, Type II or Type III type of biosensor; wherein

Type I: the mono-enzyme or the bi-enzyme system is added direct on to the electrode surface; or

Type II: the mono-enzyme or the bi-enzyme system is entrapped in the osmium based redox polymer added on the top of the electrode; or

Type III: the mono-enzyme or the bi-enzyme system and the osmium based redox polymer forms sequential coatings added on top of the electrode.

7. The biosensor according to claim 6, **characterised in** that the biosensor of Type III is one of Type III a, Type III b, Type III c or Type III d, wherein

Type III a: a second coating of the mono-enzyme is coating a dried layer of peroxidase and redox hydrogel; or

Type III b: a second coating of peroxidase and redox hydrogel is coating a dried layer of the mono-enzyme; or

Type III c: a second coating of the mono-enzyme entrapped in redox hydrogel is coating a dried layer of peroxidase; or

Type III d: a second coating of peroxidase is coating a dried layer of mono-enzyme entrapped in redox hydrogel.

8. The biosensor according to any of the preceding claims, **characterised in** that the electrode is of noble metals, such as gold, silver, platinum, palladium, or carbon/graphite-based material, such as graphite, carbon paste, vitrous carbon, carbon fibres, or conducting salts, or conducting polymers

9. The biosensor according to claim 8, **characterised in** that the electrode is made of graphite.

10. Use of the biosensor according to any of claims 1 to 9, as an analytical instrument or tool for the detection or determination of freshness biomarkers or of the content of freshness biomarkers in food, such as meat from animals or fishes, or beverages.

11. Use of the biosensor according to any of claims 1 to 9, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in body flu-

ids, such as blood, urine, saliva, sweat, in medical diagnoses or in the treatment of diseases.

12. Use of the biosensor according to any of claims 1 to 9, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in microdialysates or dialysates.

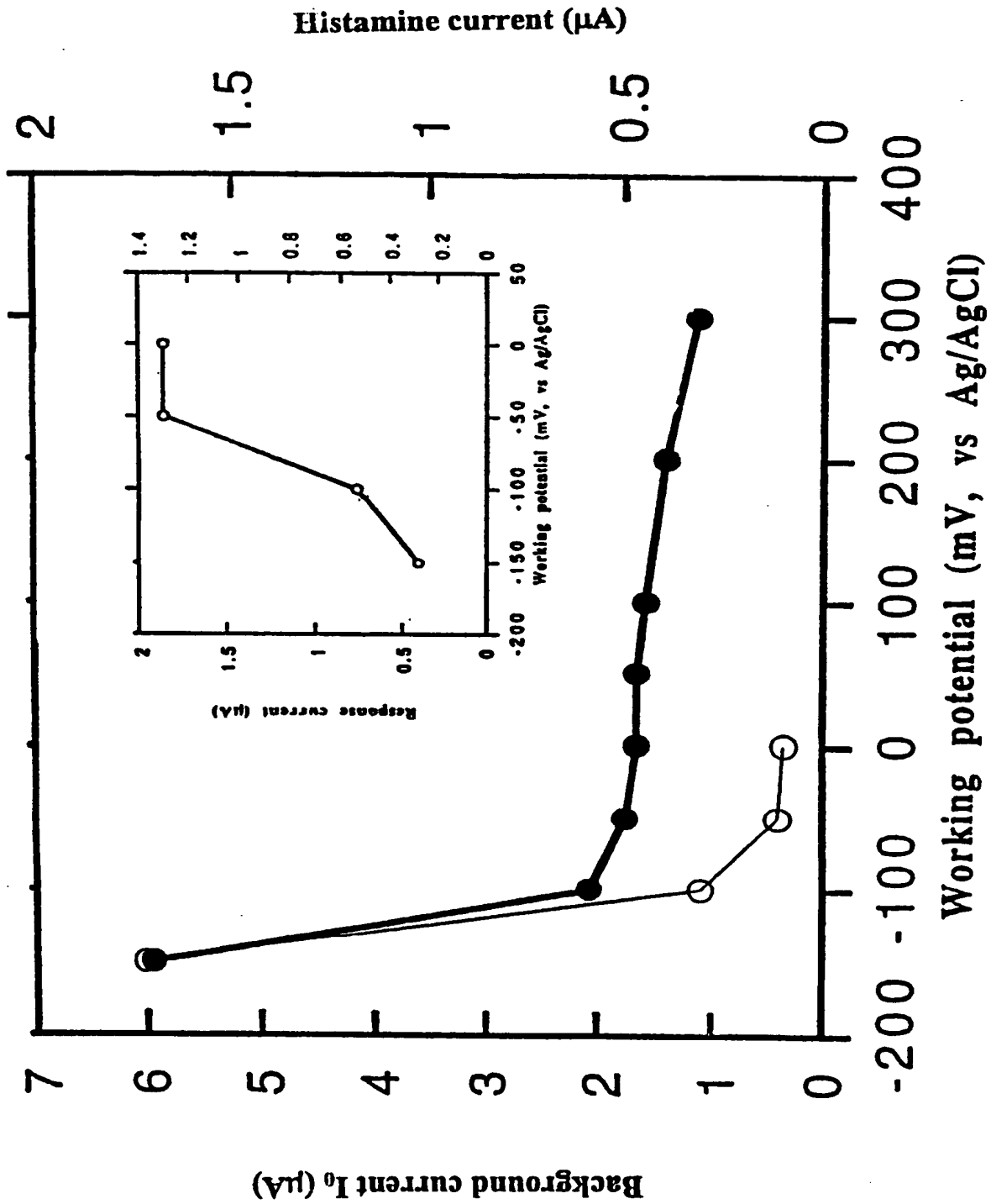

Histamine current (μA)
2
1.5
1
0.5
0
Working potential (mV, vs Ag/AgCl)
400
300
200
100
0
-100
-200
Background current I0 (μA)
7
6
5
4
3
2
1
0
Response current (μA)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Working potential (mV, vs Ag/AgCl)
50
0
-50
-100
-150
-200

Figure 1

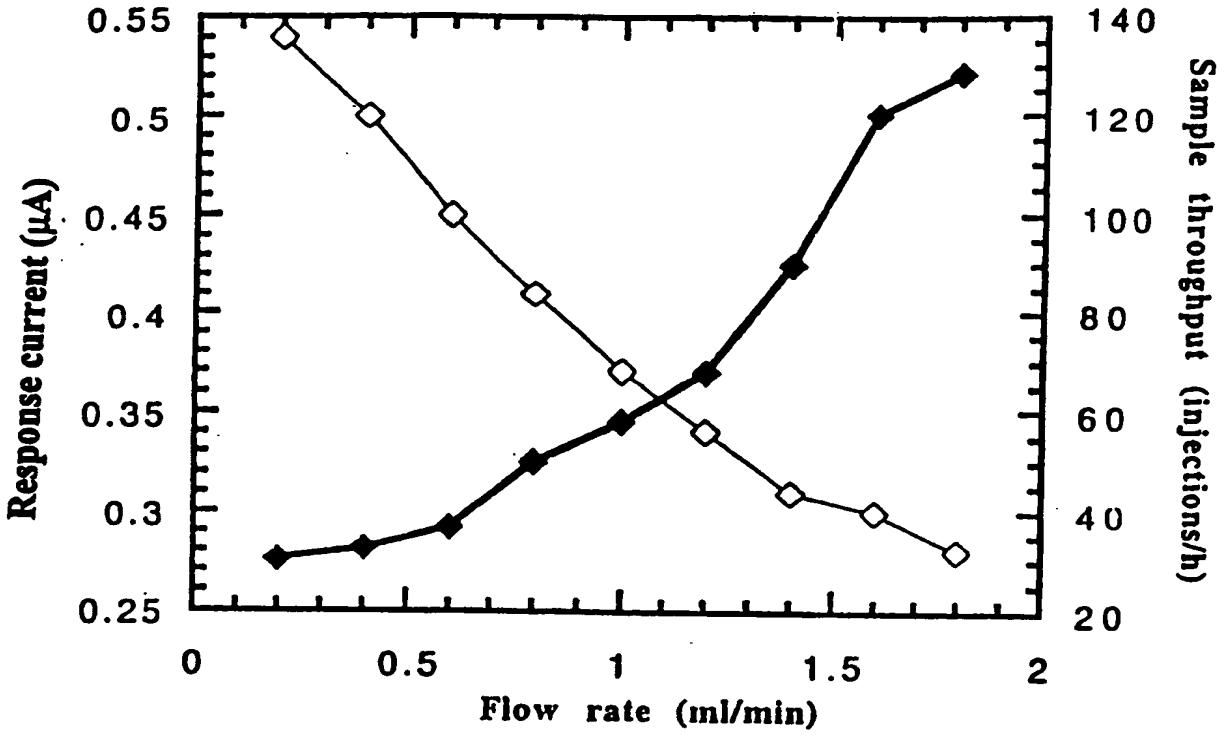


Figure 2

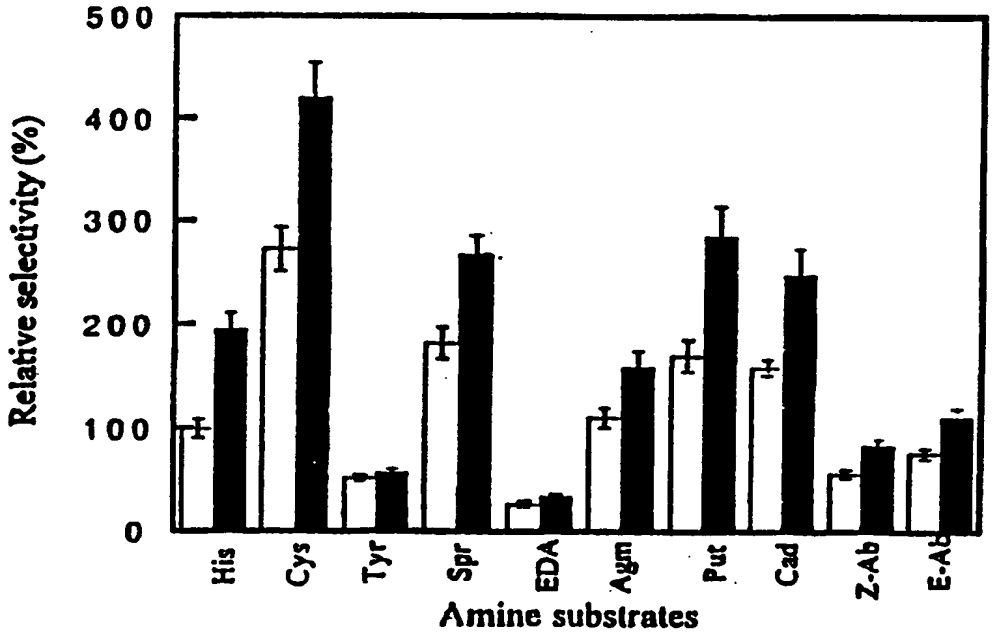


Figure 3

3/3

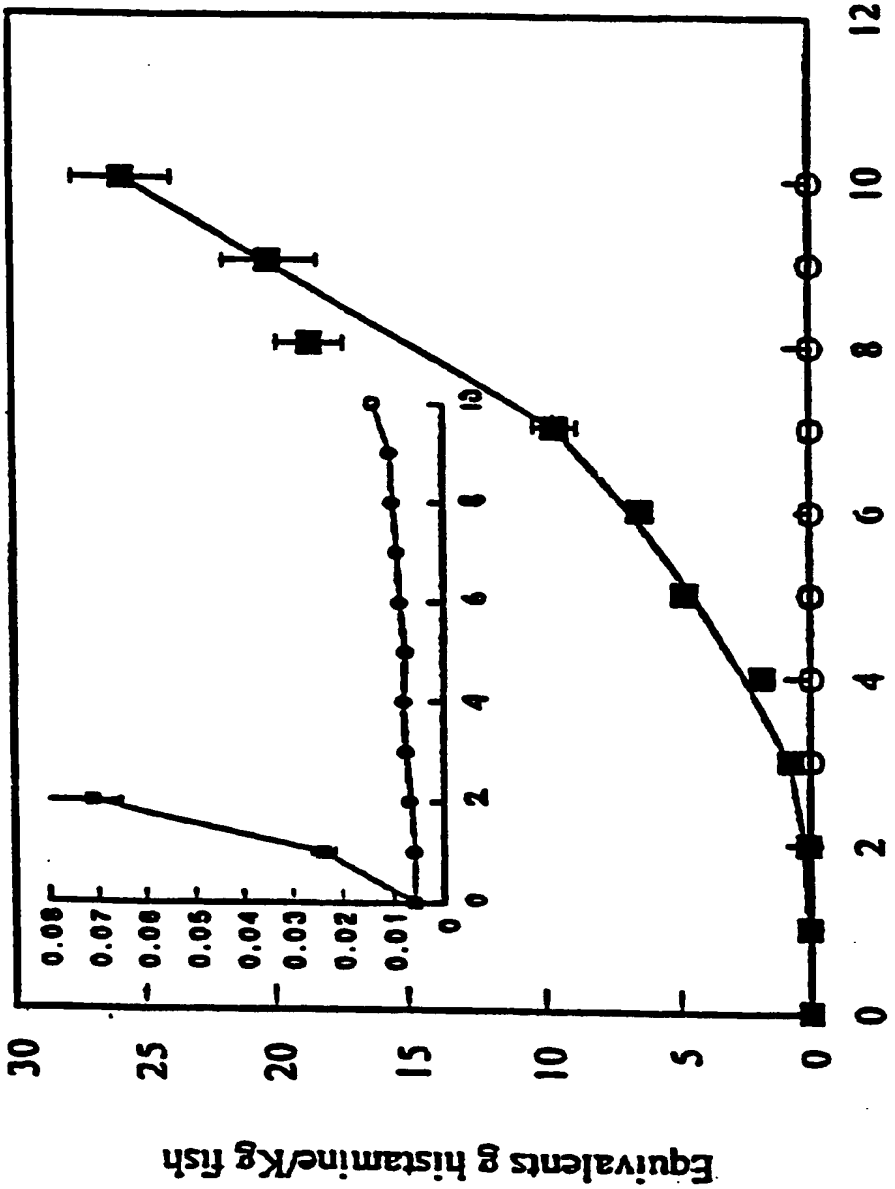

Equivalents g histamine/Kg fish
Time (days)
0
5
10
15
20
25
30
0
2
4
6
8
10
12
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08

Figure 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01449

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/26, C12Q 1/28, G01N 33/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	M.NICULESCU ET AL: Redox Hydrogel-Based Amperometric Bienzyme Electrodes for Fish Freshness Monitoring; Anal. Chem., 72 (7), pages 1591 - 1597. Web Release Date: March 4, 2000.	1-14
	--	
P,X	M.NICULESCU ET AL: Amin Oxidase Based Amperometric Biosensors for Histamine Detection;Electroanalysis 2000, 12, No. 5, pages 369 -375	1,2,5-8, 10-14
P,Y		9
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 January 2001	26 -01- 2001
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Hampus Rystedt/EÖ Telephone No. +46 8 782 25 00

Form PCT ISA 210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01449

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5565329 A (M. OHASHI ET AL), 15 October 1996 (15.10.96)	1,2,5,8, 12-14
Y		6-10
	--	
X	DIALOG(R)File 34: SciSearch (R); Accession No. 06515028. S.TOMBELLI ET AL: Electrochemical biosensors for biogenic amines: a comparison between different approaches; Analytica Chimica Acta, 1998, Vol.358, No.3 (Feb 10), pag. 277 - 284	1-5,8,10, 12-14
Y		6-10
	--	
X	P.BOUVRETTE ET AL: Amperometric biosensor for diamine using diamine oxidase purified from porcine kidney; Enzyme and Microbial Technology, vol. 20, pag. 32 - 38	1,2,8,10-14
Y		6-10
	--	
X	Sensors and Actuators B, Volume 32, 1996, G.C. Chemnitius et al, "Development of screen-printed enzyme electrodes for the estimation of fish quality" page 107 - page 113	1,2,5,8,10, 12-14
Y		6-10
	--	
X	JOURNAL OF FOOD SCIENCE, Volume 61, No 5, 1996, KEITH B. MALE et al, "Amperometric Biosensor for Total Histamine, Putrescine and Cadaverine using Diamine Oxidase" page 1012 - page 1016	1,2,5,8,10, 12-14
Y		6-10
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01449

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Food Chemistry, Volume 62, No 2, 1998, R. Draisci et al, "Determination of biogenic amines with an electrochemical biosensor and its application to salted anchovies" page 225 - page 232	1,2,5,8,10, 12-14
Y	--	6-10
Y	WO 9323748 A1 (E. HELLER & COMPANY), 25 November 1993 (25.11.93), page 11, line 13 - page 12, line 17, page 9, lines 14-15, claims	6-10
	--	
Y	US 5846702 A (ZHI DAVID DENG ET AL), 8 December 1998 (08.12.98), see abstract, column 1, lines 14-29 and claims	6-9
	--	
Y	US 5378628 A (MICHAEL GRÄTZEL ET AL), 3 January 1995 (03.01.95), see abstract, table 1	6-9
	--	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE00/01449

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE00/01449

According to Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. The present application relates to six such groups of inventions, namely:

1. An enzymatic biosensor for determining histamine concentrations using a mono-enzyme system and use thereof, according to claims 1, 2 and 5-14 (all partially).
2. An enzymatic biosensor for determining histamine concentrations using a bi-enzyme system and use thereof, according to claims 1-14 (all partially).
3. An enzymatic biosensor for determining histamine concentrations in which the enzymes are immobilised in an osmium based redox polymer and use thereof, according to claims 6-14 (all partially).
4. An enzymatic biosensor for determining histamine concentrations in which the enzymes are immobilised on the electrode surface and use thereof, according to claim 8-14 (all partially).
5. An enzymatic biosensor for determining histamine concentrations in which the enzymes and an osmium based redox polymer form sequential coatings on the top of the electrode and use thereof, according to claims 8-14 (all partially).
6. An enzymatic biosensor for determining histamine concentrations using an electrode made of noble metals, carbon or conducting salts or polymers and use thereof, according to claims 10-14 (all partially).

The technical feature common to all six inventions is a biosensor comprising an electrode and an enzyme system for detecting or determining biofreshness markers, e.g. histamine. This is well known in the prior art, through e.g. Male et al, Draisci et al, Chemnitius et al and Bouvrette et al cited in the preliminary search report. Consequently, this feature can not constitute the special technical feature required by Rule 13.2.

INTERNATIONAL SEARCH REPORT

Information on patent family members

27/12/00

International application No.
PCT/SE 00/01449

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5565329 A	15/10/96	JP 2717745 B	25/02/98
		JP 5260993 A	12/10/93
WO 9323748 A1	25/11/93	AU 3815593 A	13/12/93
		DE 4392197 T	27/04/95
		JP 7506675 T	20/07/95
		US 5320725 A	14/06/94
US 5846702 A	08/12/98	US 5589326 A	31/12/96
US 5378628 A	03/01/95	AU 656360 B	02/02/95
		BG 96988 A	31/03/94
		CZ 9203165 A	14/04/93
		DE 69216319 D,T	03/07/97
		EP 0526602 A,B	10/02/93
		FI 924726 A	19/10/92
		NO 924020 A	16/11/92
		PL 169972 B	30/09/96
		SK 316592 A	12/04/95
		AT 147107 T	15/01/97
		AU 1221992 A	15/09/92
		CA 2080840 A,C	22/08/92
		FR 2673289 A,B	28/08/92
		HU 66200 A	28/10/94
		HU 212451 B	28/06/96
		JP 2770250 B	25/06/98
		WO 9214836 A	03/09/92

From US 5,565,329 is a method for determination of histamine concentration in a sample by determination of the decrease in dissolved oxygen (DO) known. The method involves adding a solution of an enzymatic reagent, which have a histamine oxidase activity, into an examination liquid containing the test sample and detect the sensor output signal. The analyser has a reaction cell provided with a DO electrode. The enzymatic reagent is a Cu-containing fungal amine oxidase. Which is extracted from a cellmass belonging to Aspergillus Niger cultured in a culture medium including amine as a nitrogen source. This approach is not very selective and sensitive.

Enzymatic determination of biogenic amines represents an alternative that can solve the above mentioned problems. However, most of the amino oxidase biosensors require a high operating potential (>500 mV vs. Ag/AgCl), which can lead to high background currents and low selectivity due to bias signals caused by electrochemically easily oxidisable interferences, which are always present in complex matrices, such as food or beverage.

SUMMARY OF THE INVENTION

As is clear from the description above a rapid, accurate, simple and handy analytical instrumental tool is needed for determination of food hygiene all along the food process line, starting from the source to the consumer.

With the present invention the above mentioned problems have been solved, the present invention offers a highly sensitive, selective rapid and very convenient determination and/or detection of the biomarkers in very small amounts.

Thus, the present invention relates to a biosensor for detection and/or determination of the content of freshness biomarkers in food or beverage. The biosensor comprises an electrode and a copper-containing amine oxidase derived from grass pea (AO, E. C. 1.4.3.6) in a mono-enzyme system, or in a bi-enzyme system containing said amine oxidase coupled with a peroxidase.

The mono-enzyme system comprising said copper containing amine oxidase (AO) represents one preffered embodiment of the invention. The amine oxidase may be isolated from grass pea and purified according to Šebela, M., et al, Phytochem. Anal. 1998, 9, 211-222.

Another preferred embodiment of the present invention is the biosensor comprising the bi-enzyme system comprising said copper containing amine oxidase (AO) coupled with a peroxidase (PO) such as horseradish (HRP), soybean, tobacco, sweet potato or palmtree peroxidase.

In another preferred embodiment of the present invention the mono-enzyme- or the bi-enzyme- system is crosslinked into an osmium redox polymer. The osmium-based redox polymer is preferably (PVI_{13}-dmeOs) of poly- (1-vinyl-imidazole), complexed with [Os- (4,4'-dimetylbipyridine)$_2$ Cl]$^{+/2+}$, and a crosslinking agent such as poly-(ethyleneglycol)-diglycidyl-ether (PEGDGE).

Yet another embodiment of the present invention is the use of the biosensor as an analytical tool in the determination and/or detection of the freshness biomarkers in food.

Other uses and preferred embodiments of the present invention are defined in the use-claims and the subclaims.

DETAILED DESCRIPTION OF THE INVENTION

Amine oxidase represents a class of enzymes with a ubiquitous distribution in mammals, plants and micro-organisms. However, the structure, selectivity and biological functions are very different, depending on the isolation source. Grass-pea amine oxidase, fore instance, is a copper-containing amino oxidase, which besides the metal ions also contains an organic cofactor with a quinoide structure (topa-quinone) in its catalytic site.

In methods, where an amine oxidase is used, the enzyme is converting the amine to the corresponding aldehyde, with NH_3 and H_2O_2 release, according to reaction I

AMENDED CLAIMS

[received by the International Bureau on 7 March 2001 (07.03.01);
original claims 1-14 replaced by amended claims 1-12 (3 pages)]

1. A biosensor for the detection and/or the determination of freshness biomarkers, such as biogenic amines, comprising an electrode and a mono-enzyme system of an amine oxidase or a bi-enzyme system of an amine oxidase and a peroxidase, **characterised in** that the amine oxidase is a copper-containing amine oxidase derived from grass pea (AO, E.C. 1.4.3.6).

2. The biosensor according to claim 1, **characterised in** that the bi-enzyme system contains said copper-containing amine oxidase derived from grass pea coupled with horseradish, soy-bean, tobacco, sweet potato or palmtree peroxidase.

3. The biosensor according to claim 2, **characterised in** that the peroxidase is horseradish peroxidase.

4. The biosensor according to any of the preceding claims, **characterised in** that the mono-enzyme or the bi-enzyme system is crosslinked into an osmium based redox polymer.

5. The biosensor according to claim 4, **characterised in** that the osmium based redox polymer includes poly(1-vinyl-imidazole) complexed with $[Os(4,4'\text{-dimetyl-bi-pyridin})_2\ Cl]^{+/2+}$ and poly(etyleneglycol)diglycidyl-ether, as the crosslinking agent.

6. The biosensor according to any of the preceding claims, **characterised in** that the biosensor is of Type I, Type II or Type III type of biosensor; wherein

Type I: the mono-enzyme or the bi-enzyme system is added direct on to the electrode surface; or

Type II: the mono-enzyme or the bi-enzyme system is entrapped in the osmium based redox polymer added on the top of the electrode; or

Type III: the mono-enzyme or the bi-enzyme system and the osmium based redox polymer forms sequential coatings added on top of the electrode.

7. The biosensor according to claim 6, **characterised in** that the biosensor of Type III is one of Type III a, Type III b, Type III c or Type III d, wherein

Type III a: a second coating of the mono-enzyme is coating a dried layer of peroxidase and redox hydrogel; or

Type III b: a second coating of peroxidase and redox hydrogel is coating a dried layer of the mono-enzyme; or

Type III c: a second coating of the mono-enzyme entrapped in redox hydrogel is coating a dried layer of peroxidase; or

Type III d: a second coating of peroxidase is coating a dried layer of mono-enzyme entrapped in redox hydrogel.

8. The biosensor according to any of the preceding claims, **characterised in** that the electrode is of noble metals, such as gold, silver, platinum, palladium, or carbon/graphite-based material, such as graphite, carbon paste, vitrous carbon, carbon fibres, or conducting salts, or conducting polymers

9. The biosensor according to claim 8, **characterised in** that the electrode is made of graphite.

10. Use of the biosensor according to any of claims 1 to 9, as an analytical instrument or tool for the detection or determination of freshness biomarkers or of the content of freshness biomarkers in food, such as meat from animals or fishes, or beverages.

11. Use of the biosensor according to any of claims 1 to 9, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in body flu-

ids, such as blood, urine, saliva, sweat, in medical diagnoses or in the treatment of diseases.

12. Use of the biosensor according to any of claims 1 to 9, as an analytical instrument or tool for the detection or determination of biogenic amines, preferably histamine, in microdialysates or dialysates.